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PROCEEDINGS OF THE BRITISH PHARMACOLOGICAL SOCIETY

EDINBURGH

12th to 15th July, 1961

COMMUNICATIONS

W. C. Bowman and **M. J. Rand** (*Department of Pharmacology, School of Pharmacy, London, W.C.*1).
Failure of neuromuscular transmission produced by a false transmitter.

J. Raventos (*Pharmaceuticals Division, Imperial Chemical Industries, Macclesfield, Cheshire*).
The action of the optically active isomers of adrenaline and noradrenaline on the innervated vas deferens.

W. C. Bowman and **C. Raper** (*Department of Pharmacology, School of Pharmacy, London, W.C.*1).
The effect of adrenaline on slow-contracting skeletal muscles.

W. C. Bowman, B. A. Callingham and **A. A. J. Goldberg** (*Department of Pharmacology, School of Pharmacy, London, W.C.*1).
The neuromuscular blocking action of carbolonium bromide.

J. D. Harry (*introduced by* **G. Brownlee**) (*Department of Pharmacology, University of London King's College, London, W.C.*2).
The site of action of drugs on the circular muscle strip from the guinea-pig ileum.

G. Paterson (*introduced by* **G. Brownlee**) (*Department of Pharmacology, University of London King's College, London, W.C.*2).
The action of sympathomimetic amines on denervated skeletal muscle.

M. Martin-Smith, S. Nanjappa, S. Reid and **J. J. Lewis** (*Department of Experimental Pharmacology, Institute of Physiology, The University, Glasgow, W.*2).
Pharmacological activity in some thionaphthenylenes.

G. Van Petten and **J. J. Lewis** (*Department of Experimental Pharmacology, Institute of Physiology, The University, Glasgow, W.*2).
The effect of amphetamine and some related compounds upon the adenosine nucleotides of rat brain.

A. M. Barrett and **M. A. Stockham** (*introduced by* **G. A. H. Buttle**) (*Department of Pharmacology, School of Pharmacy, London, W.C.*1).
The effect of stress on circulating corticosterone levels in the rat.

R. B. Barlow and **J. T. Hamilton** (*Pharmacological Laboratory, University of Edinburgh*).
The activity of some analogues of nicotine on synaptic transmission.

D. J. Boullin, D. Mackay, P. B. Marshall and **J. F. Riley** (*Department of Pharmacology and Therapeutics, Queen's College, Dundee*).
The effect of vitamin B_6 deficiency on tumour growth and urinary histamine excretion in rats bearing a transplantable hepatoma.

Jean Eperon, Lalitha Kameswaran, Eva Kovacs and **G. B. West** (*Department of Pharmacology, School of Pharmacy, London, W.C.*1).
Histidine decarboxylase and tumour growth.

G. A. H. Buttle and **Jean Eperon** (*Department of Pharmacology, School of Pharmacy, London, W.C.*1).
Antigenic responses of normal and malignant tissue.

I. H. M. Main (*introduced by* **H. M. Adam**) (*Pharmacological Laboratory, University of Edinburgh*).
Histamine and gastric secretion in the rat.

A. K. Armitage and **J. R. Vane** (*Department of Surgery, King's College Hospital, and Department of Pharmacology, Royal College of Surgeons of England, London, W.C.*1).
The assay of catechol amines on the isolated rat stomach strip.

M. D. Day and **M. J. Rand** (*May & Baker, Dagenham, Essex, and Department of Pharmacology, School of Pharmacy, London, W.C.*1).
Sympathetic cholinergic nerves revealed by guanethidine.

G. Boyd, J. S. Gillespie and **B. R. Mackenna** (*Department of Physiology, The University, Glasgow, W.*2).
The action of guanethidine and bretylium on the response of the small and large intestine of the rabbit to stimulation of the extrinsic autonomic nerves.

Rosemary Cass and **T. L. B. Spriggs** (*introduced by* **G. B. West**) (*Department of Pharmacology, School of Pharmacy, London, W.C.*1).
Some effects of guanethidine and bretylium in the rat.

A. L. A. Boura and **A. F. Green** (*The Wellcome Research Laboratories, Langley Court, Beckenham, Kent*).
Effects of bretylium and guanethidine on responses to different rates of sympathetic nerve stimulation.

M. D. Day (*introduced by* **R. Wien**) (*May & Baker, Dagenham, Essex*).
Dopamine reversal of the blocking action of guanethidine and bretylium.

J. R. Vane (*Department of Pharmacology, Royal College of Surgeons of England, London, W.C.*1).
A general hypothesis to account for the potentiation of the effects of catechol amines by substances such as hexamethonium and bretylium.

T. C. Muir and **J. J. Lewis** (*Department of Experimental Pharmacology, Institute of Physiology, The University, Glasgow, W.*2).
The application of Ariens' theory of the evaluation of neuromuscular blocking agents.

R. P. Stephenson (*Pharmacological Laboratory, University of Edinburgh*).
Theories of the action of drugs at receptors.

S. E. Smith (*introduced by* **R. S. Stacey**) (*Department of Pharmacology and Therapeutics, St. Thomas's Hospital Medical School, London, S.E.*1).
Effect of reserpine on amine concentration in pigeon brain.

Marta Weinstock and **H. C. Stewart** (*Department of Pharmacology, St. Mary's Hospital Meaical School, London, W.*2).
Receptor similarity in different actions of analgesic drugs.

P. Withrington and **Eleanor Zaimis** (*Department of Pharmacology, Royal Free Hospital School of Medicine, London, W.C.*1).
The " reserpine " cat.

Margaret Day and **J. R. Vane** (*Department of Pharmacology, Royal College of Surgeons of England, London, W.C.*1).
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Jennifer Maclagan (*introduced by* **Eleanor Zaimis**) (*Department of Pharmacology, Royal Free Hospital School of Medicine, London, W.C.*1).
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M. W. Parkes and **A. W. Lessin** (*Roche Products, Welwyn Garden City, Herts*).
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A. W. Lessin and **R. F. Long** (*introduced by* **M. W. Parkes**) (*Roche Products, Welwyn Garden City, Herts*).
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W. H. H. Andrews and **I. del Rio Lozano** (*Department of Pharmacology, St. Mary's Hospital Medical School, London, W.*2).
Hepatic endothelial activity in the release of substances bound to plasma albumin.

B. F. Leonard (*introduced by* **R. Schneider**) (*Department of Medical Biochemistry and Pharmacology, The Medical School, Birmingham*, 15).
The pharmacology of the alkaloids of pithecolobine samanth (Benth).

J. L. Broadbent (*Smith Kline and French Research Institute, Welwyn Garden City, Herts*).
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G. R. Gough and **P. A. Robertson** (*introduced by* **R. H. Thorp**) (*Department of Pharmacology, The University of Sydney, Australia*).
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D. F. Sharman (*introduced by* **Marthe Vogt**) (*Institute of Animal Physiology, Babraham, Cambridge*).
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D. Taverner (*introduced by* **G. A. Mogey**) (*Department of Pharmacology, The School of Medicine, Leeds*, 2).
Assessment of hypnotic activity.

J. Syme (*introduced by* **W. L. M. Perry**) (*City Hospital, Edinburgh*, 10).
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R. H. Girdwood and **A. W. Dellipiani** (*Department of Medicine, University of Edinburgh*).
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S. H. Taylor, G. R. Sutherland, D. C. H. Hutchison, P. C. Robertson and **K. W. Donald** (*introduced by* **W. L. M. Perry**) (*Department of Medicine, University of Edinburgh*).
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G. P. Crean (*introduced by* **W. L. M. Perry**) (*Gastro-intestinal Unit, Western General Hospital, Edinburgh, 5, and Clinical Endocrinological Research Unit, Forrest Road, Edinburgh*).
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G. W. Ashcroft, Elizabeth McDougall and **P. A. Barker** (*introduced by* **W. L. M. Perry**) (*Royal Edinburgh Hospital for Mental Disorders, Edinburgh*, 10).
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The assessment of respiratory stimulants with particular reference to vanillic acid diethylamide.

Anne T. Lambie and **J. S. Robson** (*introduced by* **W. L. M. Perry**) (*Department of Therapeutics, Royal Infirmary, Edinburgh*).
The mechanism of the action of chlorothiazide in diabetes insipidus.

J. A. Simpson (*introduced by* **W. L. M. Perry**) (*Neurological Unit, Northern General Hospital, Edinburgh*, 5).
The use of oximes in the treatment of cholinergic crisis in myasthenia gravis.

M. Davies and **G. M. Wilson** (*Department of Pharmacology and Therapeutics, The University, Sheffield*, 10).
Aldosterone antagonists in diuretic therapy.

C. R. B. Joyce (*Department of Pharmacology, The London Hospital Medical College, London, E.*1).
Patient co-operation and the precision of clinical trials.

J. R. Hodges and **M. T. Jones** (*Department of Pharmacology, Royal Free Hospital School of Medicine, London, W.C.*1).
The determination of corticotrophin.

K. B. Holloway (*introduced by* **W. L. M. Perry**) (*Department of Anaesthetics, Royal Infirmary, Edinburgh*).
Guanethidine in controlled hypotension.

G. Onuaguluchi (*introduced by* **S. Alstead**) (*Department of Materia Medica, The University, Glasgow, W.*2).
Assessment of drug therapy in Parkinsonism.

A. H. B. Masson (*introduced by* **R. E. Lister**) (*Department of Anaesthetics, Royal Infirmary, Edinburgh*).
Some problems in the clinical evaluation of analgesia.

DEMONSTRATIONS

In the Duncan Flockhart Research Laboratories

Margaret Ettles and **R. E. Lister** (*J. F. Macfarlan & Co., Edinburgh*, 8).
Withdrawal symptoms in addicted rats.

R. E. Lister (*J. F. Macfarlan & Co., Edinburgh*, 8).
Drug modified instinctive behaviour in the gerbil.

J. E. Lightowler (*introduced by* **C. G. Haining**) (*T. & H. Smith, Edinburgh*, 11).
A method of recording respiration in laboratory animals using a body plethysmograph.

C. G. Haining and **Members of the Staff** (*T. & H. Smith, Edinburgh*, 11).
Other apparatus of general interest in pharmacology.

In the Pharmacology Department, University New Buildings

J. McC. Murdoch (*introduced by* **W. L. M. Perry**) (*Department of Infectious Diseases, University of Edinburgh*).
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K. S. Lai and **H. M. Adam** (*Departments of Medicine and Pharmacology, University of Edinburgh*).
The assay of gastrin on the perfused rat's stomach.

K. Fotherby, J. B. Brown and **J. A. Strong** (*introduced by* **W. L. M. Perry**) (*Clinical Endocrinological Research Unit, Forrest Road, Edinburgh*).
The response of the human adrenal cortex to different stimuli.

G. P. Crean (*introduced by* **W. L. M. Perry**) (*Gastro-intestinal Unit, Western General Hospital, Edinburgh, 5, and Clinical Endocrinological Research Unit, Forrest Road, Edinburgh*).
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R. H. Girdwood, J. Richmond and **J. McManus** (*Department of Medicine, University of Edinburgh*).
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W. E. Brocklehurst and **N. E. Condon** (*Department of Pharmacology, University of Edinburgh*).
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S. C. Lahiri and **W. E. Brocklehurst** (*Department of Pharmacology, University of Edinburgh*).
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S. H. Taylor, G. R. Sutherland, D. C. H. Hutchison, P. C. Robertson and **K. W. Donald** (*introduced by* **W. L. M. Perry**) (*Department of Medicine, University of Edinburgh*).
Measurements of sympatholytic action in normal and hypotensive subjects.

B. L. Ginsborg and **B. Mackay** (*introduced by* **W. L. M. Perry**) (*Departments of Anatomy and Pharmacology, University of Edinburgh*).
Structural differences in avian muscles.

M. H. Draper, H. Friebel and **K. Karzel** (*introduced by* **W. L. M. Perry**) (*Department of Physiology, University of Edinburgh*).
The action of drugs on frog sartorius muscle membrane.

J. G. Blackman and **C. Ray** (*introduced by* **W. L. M. Perry**) (*Department of Pharmacology, University of Edinburgh*).
Action of mecamylamine and pempidine at the neuromuscular junction.

J. G. Blackman, C. Ray and **B. L. Ginsborg** (*introduced by* **W. L. M. Perry**) (*Department of Pharmacology, University of Edinburgh*).
Recording from the spinal sympathetic ganglia of the frog.

R. Schneider and **B. E. Leonard** (*Department of Medical Biochemistry and Pharmacology The Medical School, Birmingham*, 15).
A simple method for the *in vivo* recording of peristalsis.

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SOME OBSERVATIONS ON PECKING IN PIGEONS

BY

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An attempt was made to analyse the pecking behaviour in pigeons induced by apomorphine. The pecking was completely suppressed by tranquillizers, barbiturates and cortisone. It was intensified by histamine, nicotine, lobeline, testosterone, progesterone and sodium taurocholate. None of the drugs tested could induce in pigeons pecking typical of apomorphine. Apomorphine induced pecking in other birds too. It was concluded that the pecking phenomenon after apomorphine is similar to the natural feeding movements performed by pigeons while eating grains and possibly it is the function of a specialized area in the limbic system of the brain which is stimulated by apomorphine.

Pecking behaviour in pigeons induced by apomorphine is described by Koster (1957) and by Dhawan & Saxena (1960a). The same phenomenon was observed by one of the authors during the study of the antiemetic properties of phenothiazine derivatives. This action of apomorphine in pigeons was thought to be an excitatory one, as intravenous administration of chlorpromazine could stop the pecking with the onset of tranquillization. Recently Dhawan & Saxena (1960a & b) reported experiments performed with a heterogenous group of drugs to elucidate the mechanism of pecking in pigeons. Since this behaviour of pigeons following apomorphine injections is not yet well understood, we are reporting some additional observations in this paper.

METHODS

Pigeons of either sex weighing from 250 to 300 g were used. There was no restriction on feeding or drinking except at the time of experiment, when both the grains and water were withdrawn. For observation of pecking each bird was kept in a separate wire mesh cage, 10 in. × 6 in. × 6 in. in size.

In the first series of experiments various drugs were tested for their effect on apomorphine pecking. For this the drugs were given half an hour before the standard dose of apomorphine, and birds were then observed for 30 min. Pecking in this period for a minimum of 10 times was taken as a positive response. In the second series various drugs were injected in pigeons to find out whether they could induce pecking activity like that seen after apomorphine. Lastly, the effect of different types of physical stress on the pecking behaviour induced by apomorphine was observed in quails, sparrows, parrots, hens, rabbits, rats, guinea-pigs, mice and dogs.

All the drugs were given intraperitoneally, and each animal was used for the next experiment only after an interval of 4 days. During each type of experiment on pigeons, 6 birds were always kept as controls.

RESULTS

It was observed that 2.0 mg/kg of apomorphine induced marked pecking activity in all the pigeons. Two pigeons out of the series showed only mild pecking activity, and these were excluded from the study. Pecking with this dose of apomorphine started in 2 to 4 min and continued throughout the 30 min period of observation. It was always preceded by some degree of excitement.

Three groups of drugs, namely, the phenothiazine derivatives, barbiturates, cortisone and nicotine, completely inhibited pecking (Table 1). In the phenothiazine group of drugs, triflupromazine was found to be the most powerful drug in suppressing apomorphine pecking and was also the most potent tranquillizing agent. Surprisingly, promethazine was found to be a persistent emetic in pigeons. Emesis started within 5 min after the injection of promethazine and usually lasted for 15 to 25 min. Emetic activity and its severity increased with the dose, and the dose which was 100% emetic was also 100% antipecking. Table 1 shows the emetic and antipecking activity of promethazine in different doses.

TABLE 1

ANTIPECKING EFFECT OF DRUGS ON THE APOMORPHINE-INDUCED PECKING IN PIGEONS

Apomorphine 2 mg/kg was given half an hour after the injection of test drug

No. of pigeons	Drug	Dose mg/kg	% Antipecking activity	% vomiting
5	Triflupromazine	2·0	20	—
5		4·0	40	—
5		6·0	80	—
5		8·0	100	—
6	Chlorpromazine	6·0	—	—
6		8·0	83	—
6		10·0	100	—
6	Prochlorperazine	16·0	—	—
6		24·0	50	—
6		28·0	83	—
6		32·0	100	—
5	Promethazine	6·0	20	20
5		10·0	40	40
5		20·0	60	60
5		40·0	60	100
5		60·0	100	100
4	Pentobarbitone	8·0	25	—
4		16·0	100	—
4	Amylobarbitone	8·0	25	—
4		16·0	100	—
4	Cortisone	4·0	25	—
4		8·0	75	—
4		16·0	100	—
2	Nicotine	4·0	100	100

The other drugs which blocked the pecking activity were pentobarbitone, amylobarbitone, cortisone and nicotine (Table 1). The first three drugs suppressed pecking completely in doses of 16 mg/kg, while nicotine was effective as an antipecking agent only in a dose of 4 mg/kg, a dose which produced vomiting in all the pigeons. In lower doses nicotine apparently intensified the apomorphine pecking in pigeons (Table 2).

Like nicotine, some of the drugs also intensified the apomorphine pecking. Although it was not possible to express this quantitatively it was repeatedly confirmed by the different observers. Drugs like nicotine, lobeline, testosterone, and sodium taurocholate made the apomorphine pecking so compulsive that the birds pecked incessantly and no intervals between the bouts of pecking were seen, as was noticed in control pigeons.

A search was made for a drug which would precipitate pecking like apomorphine. The drugs from different groups, for example, emetine, ouabain and copper sulphate (emetics), dexamphetamine, atropine methylphenidate, nicotine and lobeline (central nervous system stimulants), acetylcholine, neostigmine and physostigmine (parasympathomimetics), adrenaline (sympathomimetic) and insulin (hypoglycaemic), were

TABLE 2

DRUGS WHICH APPARENTLY INTENSIFIED THE APOMORPHINE PECKING IN PIGEONS

Apomorphine (2 mg/kg) given half an hour after the test drug. 1 = Excessive. 2 = Moderate. Potentiation of pecking was recorded as either 1 or 2 separately by three observers, and 1 was taken to be reliable when there was an agreement in the observations of all the observers

No. of pigeons	Drug	Dose mg/kg	Increase in pecking
4	Nicotine	0·2	2
5		0·3	1
4		0·4	2
4	Histamine	0·8	?
4		1·6	2
5	Testosterone	10·0	1
4		20·0	1
4	Oestradiol	10·0	2
5		20·0	2
4	Progesterone	20·0	1
4	Sodium taurocholate	40·0	1
4		80·0	2

given intraperitoneally in a wide range of doses. None of these induced pecking typical of apomorphine. However, nicotine, lobeline, methylphenidate and acetylcholine produced a behaviour pattern very akin to that seen after apomorphine. Some pigeons did show pecking activity after the injections of some of these drugs, but the pecking score never exceeded 10 in 30 min.

The relation of feeding to pecking was seen by starving the pigeons, by insulin injection and by giving glucose before and after the onset of pecking. Feeding sensations were artificially created by keeping the grains under a transparent glass floor over which the pigeons were kept standing. All these methods did not influence the pecking behaviour of pigeons.

The effect of certain other agents on pecking in pigeons was also observed. Anxiety and fear were created by keeping dogs or hens near the cages, by loud noise and by sudden exposure to bright light. All these, including the simple movements of an observer towards the cage from a distance, resulted in a prompt cessation of pecking activity. However, pecking started again after the removal of a disturbing factor.

Apomorphine induced pecking in other birds like quails, sparrows and parrots in the same dose, that is, 2 mg/kg. In the case of quails, pecking was positive only when the birds were grouped in one cage, while parrots exhibited only slight pecking activity. In hens, a dose of 3.0 mg/kg was required to induce pecking. In rats, mice, rabbits and guinea-pigs, apomorphine produced characteristic chewing movements. In dogs the emetic action was noted.

DISCUSSION

It is interesting to note that apomorphine, which consistently produces an emetic action in man, dogs, cats and some other animals, fails to do so in pigeons. This finding confirms that of Dhawan & Saxena (1960a) and Madjarek & Stern (1956). Instead of emesis, apomorphine consistently induces pecking behaviour in pigeons. The observations described give no satisfactory explanation for the phenomenon of pecking. However, it has been possible to analyse pecking behaviour to some extent.

Pecking does not appear to be related to the act of vomiting, as none of the emetic drugs (emetine, ouabain, copper sulphate, atropine, nicotine, promethazine) induced pecking in non-emetic doses or in emetic doses before, during or after vomiting. In fact, it is possible to dissociate emetic and pecking effects, from the observations with promethazine and nicotine, which, in emetic doses, show antipecking activity.

One possibility is that pecking is the expression of general stimulation of the nervous system, since pigeons always show some degree of excitement before they start pecking after the injection of apomorphine. This hypothesis is not tenable, since a number of known stimulant drugs failed to induce pecking.

The pecking phenomenon is also not due to a decreased excitability of the brain as drugs like acetazoleamide (Millichap, Woodbury & Goodman, 1955), azacyclonol (Dhawan & Saxena, 1960a), barbiturates and some phenothiazine derivatives do not induce pecking. The last two groups, on the contrary, exhibit strong antipecking activity.

The feeding centre too does not show any relation to pecking, as can be deduced from our observations and those of Dhawan & Saxena (1960a). Starvation or hypoglycaemia produced by excessive doses of insulin is ineffective. Pecking also remains unaffected even when glucose is given before or during the pecking activity induced by apomorphine.

It is, moreover, difficult to accept the view that pecking is a feeding "hallucination," as suggested by Koster (1957). Firstly, birds behave quite normally when they are interrupted during pecking, and pecking continues only when the pigeons are kept undisturbed. Secondly, grains were put under a glass floor in an attempt to simulate artificial "hallucination," but this procedure did not induce pecking in normal pigeons. In addition, various hallucinogenic agents (Dhawan & Saxena, 1960a) are ineffective in producing pecking. Pecking is not a sign of anxiety or fear complex, as the physical methods used to produce these do not make the pigeons peck.

All the observations described above could be explained on the assumption that there is a specialized part of the nervous system, like the chemoreceptor trigger zone of emesis (Wang & Borison, 1950), which is specifically stimulated by apomorphine. This area normally appears to control the voluntary movements of the birds, stimulating pecking, which they make while picking up the grains. When apomorphine stimulates this area there appears a compulsive voluntary feeding-like movement—a pecking phenomenon. Additional evidence is obtained from our observations to show that pecking is identical to feeding movements. Apomorphine is able to induce pecking in birds like quails, sparrows, parrots and hens which have the same type of feeding movements as pigeons while picking up the grains. Secondly, apomorphine produces chewing movements in rats (Du Toit & Christensen, 1948), mice, rabbits and guinea-pigs. Chewing movements of these animals are comparable to the pecking or feeding movements of pigeons.

No drug in this investigation and that of Dhawan & Saxena (1960a, b) could induce typical apomorphine pecking. Therefore, it appears that, for a drug to be an effective pecking agent, a selective action like apomorphine on the pecking zone with the least general effects on the central nervous system is necessary.

Lastly, it is suggested that, as pecking seems to be a behaviour pattern, an area controlling this activity is more likely to be situated in the limbic system of the brain.

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SUPPRESSION OF OUABAIN-INDUCED ATRIAL ARRHYTHMIAS BY CAROTID SINUS STIMULATION

BY

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In dogs anaesthetized with chloralose ectopic atrial arrhythmias were produced by subepicardial injection of ouabain. Stimulation of the right carotid sinus abruptly suppressed the ectopic arrhythmias. They returned on cessation of stimulation and sometimes already during the period of stimulation. It is suggested that in view of its response to carotid sinus stimulation the ouabain-induced arrhythmia resembles paroxysmal atrial tachycardia and that it is distinct from the aconitine-induced arrhythmia which on account of its response to vagal stimulation has been classified as atrial flutter.

It is well known to clinicians that attacks of atrial paroxysmal tachycardia can often be terminated by carotid sinus pressure whereas this procedure is ineffective in atrial flutter. It is equally well known that different types of atrial arrhythmias can be produced in animals by local application of various drugs to the epicardial surface of the heart (Scherf, 1944, 1947; Kisch, 1944; Scherf & Chick, 1951; Prinzmetal, Corday, Brill, Oblath & Kruger, 1952; Scherf, Blumenfeld, Taner & Yildiz, 1960), but it has never been shown that any of these resemble paroxysmal atrial tachycardia in man by the crucial test of being consistently suppressible by means of carotid sinus stimulation. It has now been found that the arrhythmia produced by subepicardial injection of ouabain is regularly susceptible to carotid sinus stimulation.

METHODS

Dogs weighing between 9 and 14 kg were premedicated with morphine, 1 mg/kg injected subcutaneously, and anaesthetized with chloralose, 0.1 g/kg injected intravenously. The blood pressure was recorded from a femoral artery and a cannula was inserted into the trachea.

In all experiments stimulation of the carotid sinus was carried out on the right side with both sinus and aortic nerves intact in order to approximate the effects obtained in man, where pressure on the right carotid sinus is more frequently effective in terminating attacks of atrial paroxysmal tachycardia (Scherf & Schott, 1953). The right carotid sinus was exposed, and stimulating shielded platinum electrodes were applied to the dissected sinus nerve and connected to a transistor stimulator (Joslin, 1959). Stimulation was carried out with impulses of 15 V, 1 msec, 100/sec. In a few experiments the innervated right carotid sinus was isolated, according to the method of Moissejeff (1927), making possible its distension by introducing liquid into it, and at the same time recording the intrasinusal pressure.

After dissection of the carotid sinus, the sternum was split, the chest opened in the midline to expose the heart, while artificial respiration was given with a Starling ("Ideal") pump.

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Lead 2 of the electrocardiogram was recorded on a direct-writing electrocardiograph of the Cambridge Instrument Co. ("Electrite"). In this lead ectopic beats originating from the site of an ouabain injection were characterized by inverted P waves and were easily distinguishable from normal sinus beats which had upright P waves.

Ouabain (Burroughs Wellcome & Co.), 0.1 ml. of a 0.1% solution in either 70% alcohol, 0.9% sodium chloride solution, or distilled water, was injected subepicardially at the posterior surface of the heart between the inferior vena cava and a pulmonary vein. Unless otherwise stated, the ouabain solution was freshly prepared, as advised by Scherf (1944), in order to obtain arrhythmia consistently.

RESULTS

Seven experiments were carried out with subepicardial injection of freshly prepared ouabain solution. In four experiments, arrhythmias with inverted P waves were produced; in two, the arrhythmia was doubtful because the P waves closely resembled the normal sinus P waves, and in one atrial fibrillation occurred after a period of ventricular arrhythmia. In three different experiments, two subepicardial injections of 0.1 ml. of distilled water, four of 70% alcohol and one injection of 10% sodium chloride solution did not produce any ectopic arrhythmia.

In the four experiments in which the ouabain produced ectopic arrhythmia with inverted P waves, the arrhythmia was temporarily abolished by stimulation of the right carotid sinus nerve. The stimulation caused each time a prompt and appreciable fall in arterial blood pressure. The arrhythmia returned either during the period of stimulation or after its cessation, in which case it was usually preceded by a few sec of undisturbed sinus rhythm. When the sinus nerve was stimulated again, the arrhythmia was again temporarily suppressed. This occurred twice in one, and four, seven and ten times in the other three experiments.

One of the four experiments is illustrated in Fig. 1. The tracing (a) shows the effect of sinus nerve stimulation before the injection of ouabain. Stimulation produced a gradual slowing of the heart; the rate also returned gradually after the end of stimulation. These gradual changes are characteristic of the effect of sinus stimulation on the normal rhythm, and contrast with the abrupt changes brought about during ectopic arrhythmia produced by ouabain, as shown in (b). The ectopic arrhythmia was fully developed 4 min after the ouabain injection. When the sinus nerve was stimulated for periods of about 20 sec the ectopic rhythm was temporarily suppressed. Such stimulation was carried out seven times. The tracing (b) shows the effect of the third period of stimulation. At the beginning of Fig. 1 (b) the ectopic atrial arrhythmia has cycle lengths varying between 0.24 and 0.32 sec (rate 188 to 250). The ventricular complexes have a pattern which suggests injury, presumably because ouabain leaked into the ventricular myocardium. There was in addition alternation in cycle length, a phenomenon previously observed in atrial arrhythmia elicited by aconitine and in ventricular tachycardia precipitated by ouabain (Scherf, 1944; Scherf & Terranova, 1949). Stimulation of the carotid sinus nerve abruptly suppressed the ectopic arrhythmia and a slow sinus rhythm appeared. The third beat after the onset of stimulation was no longer ectopic, its P wave being upright. The onset of stimulation was recorded manually and with appreciable error. Therefore the length of the interval between onset of stimulation, as indicated on the record, and suppression of the ectopic arrhythmia cannot

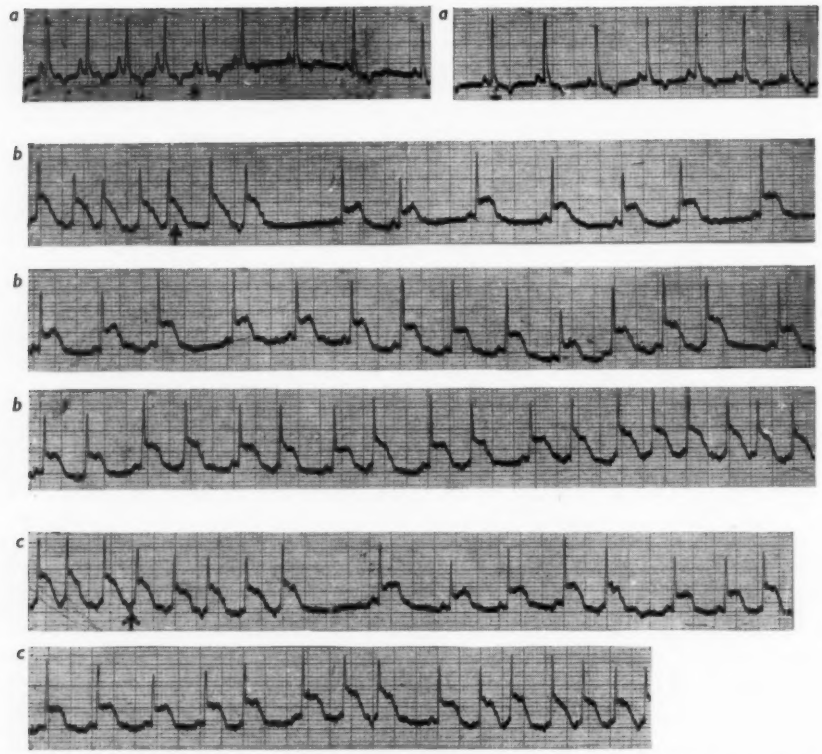

Fig. 1. Electrocardiogram of a dog anaesthetized with chloralose 100 mg/kg after premedication with morphine 1 mg/kg. Lead 2. (a) Stimulation of the right carotid sinus nerve between the arrows. The interval between the two sections of the record was 10 beats. (b) About 5 min after a subepicardial injection of 0.1 ml. of a 0.1% solution of ouabain. The three sections of the record are continuous. From the arrow until the end of the record the right carotid sinus nerve was stimulated. (c) About half a min after (b). The two sections of (c) are continuous. From the arrow until the end of the record renewed stimulation of the right carotid sinus nerve. Time base 0.2 sec between the thick vertical lines.

be regarded as accurate. In this experiment the atrial arrhythmia returned during the period of stimulation. The first signs were single atrial extrasystoles. The first extrasystole, which occurred after 19 sinus beats, is reproduced as the penultimate beat in the middle strip; it is characterized by its prematurity and inverted P wave. The beginning of the bottom strip of (b) shows that each of the first five sinus beats was followed by one atrial extrasystole (atrial bigeminy). The interval between each extrasystole and its preceding sinus beat was constant (0.38 sec), that is, the extrasystoles had fixed coupling. The end of the bottom strip illustrates the reappearance of the atrial arrhythmia 17.5 sec after the beginning of stimulation. Fig. 1 (c) shows the effect of the fourth period of stimulation to demonstrate the renewed suppression of the atrial arrhythmia, but this time the effect was unusually

transient. The first single atrial extrasystole appeared already after four sinus beats, the shortest duration of converted undisturbed sinus rhythm during stimulation found in any experiment. Further single atrial extrasystoles with fixed coupling occurred after two and four sinus beats, then two extrasystoles followed the next sinus beat. Finally, about 10 sec after the beginning of stimulation, the ectopic atrial arrhythmia recurred, as shown at the end of the figure. After the seventh stimulation of the carotid sinus nerve the right vagus was cut. Stimulation of its peripheral end for 24 sec resulted in cardiac standstill with ventricular escape beats, followed after the end of stimulation by sinus rhythm. A second injection of ouabain produced again ectopic atrial arrhythmia ; five consecutive stimulations of the carotid sinus nerve had no effect on the ectopic arrhythmia, whereas subsequent stimulation of the peripheral end of the right vagus again precipitated cardiac standstill with escape beats.

Fig. 2 illustrates the experiment in which ten consecutive stimulations of the right carotid sinus nerve suppressed atrial ectopic arrhythmias throughout the period of stimulation which lasted for 8 to 14 sec. The tracing (a) taken before the

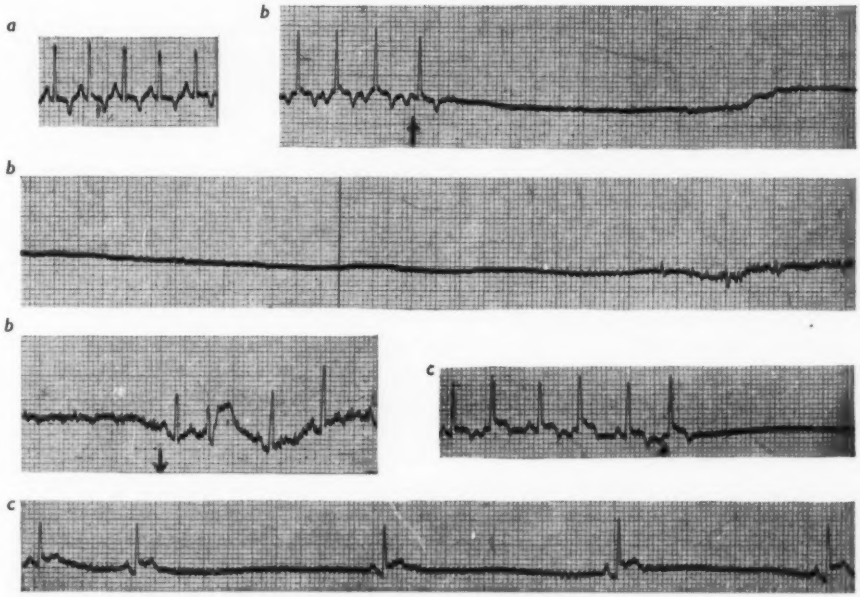

Fig. 2. Electrocardiogram of a dog anaesthetized with chloralose 100 mg/kg after premedication with morphine 1 mg/kg. Lead 2. (a) Before, (b) 4 min after subepicardial injection of 0.1 ml. of a 0.1% solution of ouabain. Between the arrows first stimulation of the right carotid sinus nerve. The three sections of (b) are continuous. (c) 10 min after the ouabain injection. From the arrow till the end of the record stimulation of the right carotid sinus nerve (seventh period of stimulation). The two sections of (c) are continuous. Time base 0.2 sec between the thick vertical lines.

ouabain injection shows sinus rhythm of a rate of about 214/min. Half a minute after the injection single atrial extrasystoles occurred. This was unusually early, since according to Scherf (1944) it takes as a rule several min for subepicardially injected digitalis glycosides to produce ectopic arrhythmia. Two min after the injection, periods of ectopic atrial arrhythmia of increasing length started to alternate with periods of sinus rhythm, both at first at a rate of about 150. Four min after the injection the ectopic atrial arrhythmia was fully developed and its rate had increased. After a series of 21 consecutive atrial ectopic beats with cycle lengths of 0.32 to 0.35 sec (rate 171 to 187) the carotid sinus nerve was stimulated. This produced cardiac standstill throughout the 12 sec period of stimulation. The beginning of tracing (b) shows the last four ectopic atrial beats with deeply inverted P waves before the onset of stimulation which resulted in the cardiac standstill. On cessation of stimulation there was return of sinus rhythm, the first sinus beat being followed by a ventricular extrasystole. The sinus rhythm had about the same rate as the previous ectopic arrhythmia. Such similarity of rates between sinus and an ectopic rhythm has previously been observed in ectopic ventricular arrhythmias elicited by the subepicardial injection of veratrine, sodium oxalate, citrate and chloride (Sche ., Blumenfeld, Golbey, Ladopoulos & Roth, 1954). The period of sinus rhythm lasted for 41 beats. It was followed by ectopic atrial arrhythmia, which was again abruptly suppressed with renewed carotid sinus nerve stimulation, and throughout its duration (11.5 sec) the ectopic arrhythmia was replaced by a slow sinus rhythm of a rate of 34 to 37/min (not shown in Fig. 2).

Essentially the same sequence of events was observed during all subsequent periods of stimulation. There was each time abrupt suppression of the ectopic rhythm during stimulation, followed either by cardiac standstill with or without an occasional idioventricular escape beat, or by a slow sinus rhythm (sinus escape), and after cessation of stimulation the ectopic arrhythmia returned, preceded by a period of undisturbed sinus rhythm. This ectopic arrhythmia consisted exclusively of ectopic beats as illustrated in Fig. 2b, or was an atrial bigeminy, as illustrated in Fig. 2c, which reproduces the effect of the seventh stimulation carried out 10 min after the ouabain injection. The beginning of Fig. 2c shows three sinus beats each followed by an atrial extrasystole. On stimulation this arrhythmia was immediately replaced by a slow sinus rhythm.

In a few experiments the ouabain solution used for subepicardial injection was not freshly prepared, but a stock solution about a year old containing 0.8 mg anhydrous ouabain in 1 ml. of 70% alcohol was used. In only one of them did the injection result in ectopic atrial arrhythmia. In this experiment, instead of stimulating the right carotid sinus nerve, the isolated innervated right carotid sinus was distended to a pressure of 200 mm Hg for periods varying between 13 and 43 sec. Each of nine successive distensions resulted in abrupt suppression of the atrial ectopic arrhythmia ; in six of these, single atrial extrasystoles appeared during the period of distension, but again only after an initial undisturbed sinus rhythm. As in the experiment of Fig. 1 the extrasystoles had fixed coupling. Fig. 3a illustrates the effect of the fifth period of distension. The first atrial extrasystole during the distension occurred after ten sinus beats, and the coupling of the three extrasystoles at the end of the tracing was 0.28 sec. This tracing also illustrates the strong vagal

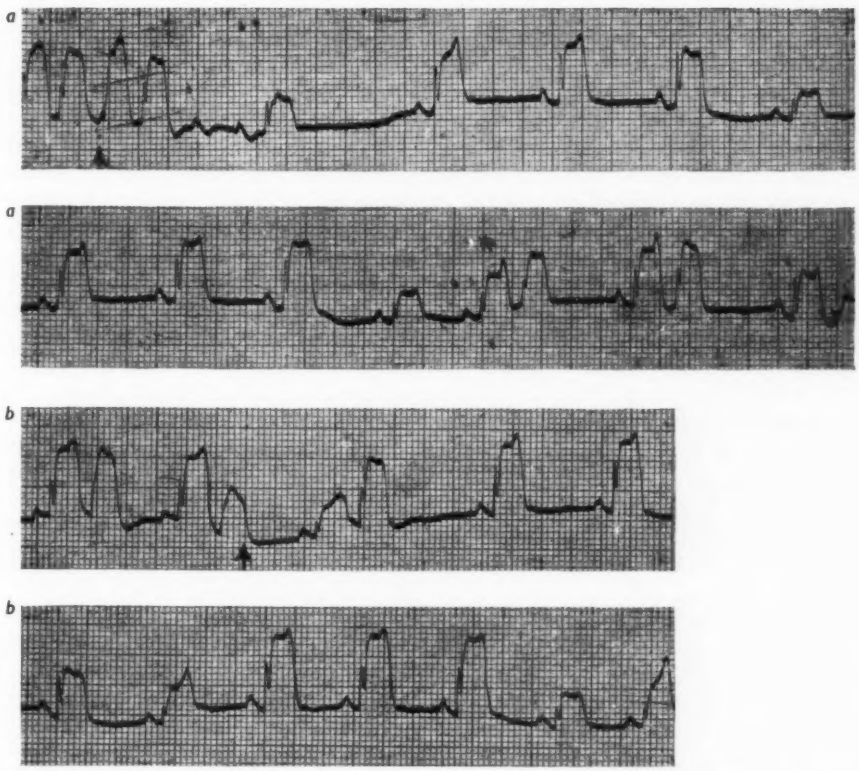

Fig. 3. Electrocardiogram of a dog anaesthetized with chloralose 100 mg/kg after premedication with morphine 1 mg/kg after subepicardial injection of 0.1 ml. of a 0.1% solution of ouabain. Lead 2. In both (a) and (b), which are both continuous records, the innervated right carotid sinus was distended to a pressure of 200 mm Hg from the arrow until the end of the record; (a) fifth and (b) sixth period of distension 5 and 6 min respectively after the injection. Time base 0.2 sec between the thick vertical lines.

effect on atrio-ventricular conduction immediately on suppression of the ectopic arrhythmia, as shown by the fact that the first sinus P wave was not followed by a ventricular complex and the second only after a prolonged P-R interval of 0.18 sec compared with 0.12 to 0.14 sec, the lengths of the P-R intervals of the subsequent sinus beats. After the distension the rhythm became that of atrial bigeminy, and renewed distension again selectively suppressed the extrasystoles. This is shown in tracing (b), in which the two sinus beats before and the first sinus beat after the onset of distension were each followed by an atrial extrasystole with a fixed coupling of 0.28 sec. The subsequent undisturbed sinus rhythm had a rate of about 94/min. At the end of distension atrial bigeminy returned. Selective suppression of the extrasystoles was again obtained during the two subsequent periods of renewed distension. It is interesting in this connexion to recall a similar observation of

Scherf (1944) made on arrhythmias induced by topical application of digitalis glycosides. He found a temporary suppression of atrial extrasystoles in atrial bigeminy by stimulation of the right vagus.

DISCUSSION

The finding that the atrial ectopic arrhythmia and atrial extrasystoles elicited by subepicardial injection of ouabain were consistently and abruptly suppressed by right carotid sinus nerve stimulation or by distension of the isolated innervated right carotid sinus is relevant to the problem of the relation between atrial flutter and paroxysmal atrial tachycardia. These two forms of arrhythmia are now generally regarded as being due to rapid stimuli originating in an ectopic atrial centre, whereas formerly atrial flutter was attributed to a circus movement of the excitation wave in the atria, in accordance with Lewis's theory. This change in view about the origin of flutter results from the observation that the atrial arrhythmia elicited in dogs by the topical application of aconitine to the atrial surface responded to vagal stimulation by an increase in atrial rate and occasional conversion into atrial fibrillation (Scherf, 1947 ; Scherf, Romano & Terranova, 1948 ; Scherf, Schaffer & Blumenfeld, 1953). Many years earlier the same vagal response was obtained during atrial flutter produced by faradization (Rothberger & Winterberg, 1914). Suppression of the atrial arrhythmia by vagus stimulation in the two conditions was only infrequently obtained (Lewis, Drury & Bulger, 1921 ; Scherf & Terranova, 1949 ; Scherf, Blumenfeld, Mueller & Beinfield, 1953). It was on the basis of these observations that the aconitine-induced arrhythmia was classified as atrial flutter as distinct from paroxysmal tachycardia. The concept of a focal origin has also been applied to atrial flutter in man (Puech, 1956). The mode of origin of atrial arrhythmias, a subject which is still controversial (see Rosenblueth & Garcia Ramos, 1947 ; Brown & Acheson, 1952 ; Lanari, Lambertini & Ravin, 1956), has been reviewed by Scherf & Schott (1953), Spang (1957), Holzmann (1960), and Katz & Pick (1960).

The assumption that both arrhythmias, atrial flutter and paroxysmal tachycardia, are due to rapid stimulus formation in an ectopic centre presupposes a closely allied mode of origin. In fact, Prinzmetal *et al.* (1952) tentatively suggested a unitary nature of the two arrhythmias. However, there are serious objections to such a view. Atrial tachycardia is often found in subjects with otherwise normal hearts, whereas the reverse is true of atrial flutter. Pressure on the carotid sinus often abruptly terminates an attack of paroxysmal atrial tachycardia, but this does not occur in patients with atrial flutter.

The abrupt suppression by carotid sinus stimulation of the atrial ectopic arrhythmias induced in dogs by subepicardial injection of ouabain resembles the abrupt termination by carotid sinus pressure of paroxysmal atrial tachycardia in man. But, in contrast to the permanent suppression usually obtained in man, the effect on the ouabain arrhythmia in dogs was transient. This is perhaps not surprising, owing to the persistence of the arrhythmogenic agent ouabain. The transient effect of vagus stimulation on the flutter induced by topical application of aconitine has been similarly explained (Scherf, Schaffer & Blumenfeld, 1953).

The fact that the ouabain-induced arrhythmia was suppressed by carotid sinus stimulation whereas the arrhythmia induced by aconitine responds differently, that is, by an increase in atrial rate and occasionally by conversion into atrial fibrillation, suggests a difference in nature of these two arrhythmias. This conclusion rests on the assumption that the efferent pathway of the carotid sinus reflex to the heart is the vagus and that extravagal components play no or only an insignificant part in the anti-arrhythmic effect of carotid sinus stimulation. The findings of Winder (1938) that in dogs, with the major secondary factors controlled, the maximum extravagal component of carotid pressoreceptive slowing of normal rhythm was only approximately 8% may be relevant in this connexion. On account of its response to carotid sinus stimulation the ouabain-induced arrhythmia should be considered as resembling paroxysmal atrial tachycardia, as distinct from the aconitine-induced arrhythmia whose response to vagal stimulation characterized it as atrial flutter.

I wish to express my gratitude to the Charing Cross Hospital Medical School for affording me the facilities to carry out this work, and in particular to Dr J. B. E. Baker for his invaluable help without which these experiments could not have been performed.

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COMPARISON OF THE FUNCTIONAL EFFECTS OF DYFLOS, TRI-*o*-CRESYL PHOSPHATE AND TRI-*p*-ETHYLPHENYL PHOSPHATE IN CHICKENS

BY

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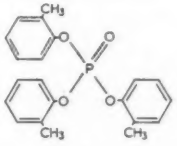
Tri-*p*-ethylphenyl phosphate is unique amongst the organophosphorus compounds which produce neurotoxic effects in not being an inhibitor of cholinesterase. The dysfunction it produces is also marked by some unusual features. Thus it produces a characteristic high-stepping gait which develops at varying periods after intramuscular injection but more regularly following oral administration. A careful comparison of the character, onset and development of the effects of diisopropyl phosphorofluoridate (dyflos), tri-*o*-cresyl phosphate and tri-*p*-ethylphenyl phosphate has illustrated the differences between the two former substances and tri-*p*-ethylphenyl phosphate. It has also confirmed a previous suggestion that this substance acts in a different manner from the other two, a suggestion supported by the histological evidence.

Despite marked differences in chemical structure certain triaryl phosphates, notably tri-*o*-cresyl phosphate (I), and some alkyl organophosphorus compounds, for example, dyflos (II), are known to produce closely similar neurotoxic effects in the hen (Barnes & Denz, 1953).



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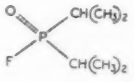

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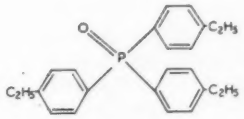

III

Recently it has been reported that tri-*p*-ethylphenyl phosphate (III) also possesses neurotoxic properties (Bondy, Field, Worden & Hughes, 1960), despite the fact that it is the only known triaryl phosphate with these properties which does not possess at least one phenyl group with a substituent in the ortho position. However, Silver (1960) has pointed out that the functional disturbance produced by this compound has some additional characteristics which further stress its anomalous position. In addition it has been claimed by Aldridge & Barnes (1961) that tri-*p*-ethylphenyl phosphate shows no significant anticholinesterase activity either *in vitro* or *in vivo*.

In an attempt to resolve the problems posed by these observations it was considered that a thorough comparison of the functional disturbances produced by tri-*o*-cresyl phosphate, dyflos and tri-*p*-ethylphenyl phosphate was necessary.

At the same time histological comparisons were made of the distribution of the lesions caused by these substances in the expectation that such differences might explain differences in functional effect.

METHODS

Tri-*o*-cresyl phosphate was obtained commercially from Kodak, but tri-*p*-ethylphenyl phosphate and dyflos were prepared at the Chemical Defence Experimental Establishment by Mr M. J. Rumens. Their purity, as judged by infra-red analysis, was not less than 95%. Tri-*o*-cresyl phosphate and tri-*p*-ethylphenyl phosphate were used undiluted, but dyflos was given in a 10% solution of ethanol in 0.9% sodium chloride solution. Adult hens (1 to 2 years of age), pure strain white leghorns, were used in all experiments. All agents were injected into the breast muscle 10 min after an intramuscular injection of 100 mg/kg of pralidoxime (2-hydroxyiminomethyl-*N*-methylpyridinium) methanesulphonate and 1 mg/kg atropine sulphate (Davies, Holland & Rumens, 1960).

In a separate series dyflos and tri-*p*-ethylphenyl phosphate were given orally to compare the influence of route of administration.

After the injections the birds were kept in cages for 6 days. They were then transferred to an open paddock and each was examined daily by at least two observers.

For the experiments in which the functional effects of these compounds were compared, the following rigid procedure was adopted. From the seventh day after injection, each bird was made to walk at varying rates and for varying times along a narrow earthed passage-way and the character of its gait assessed on the following scale: Slight but definite clumsiness or incoordination, 2 points; ataxia, implying a grosser degree of incoordination, but without serious incapacitation—"drunken gait," 4 points; marked ataxia with inability to maintain an upright stance for any length of time, a waddling or shuffling gait, the bird progressing on its hocks, 6 points; total inability to rise or walk, weak limb movements, 8 points.

If, particularly in the early stages, there was some doubt about the presence of incoordination, vigorous exercise generally served to resolve the difficulty. In the case of dubious signs the sign had to be at least suspected on three consecutive days before being regarded as real.

A high-stepping but often well-coordinated gait has been reported by Silver (1960) as characteristic of tri-*p*-ethylphenyl phosphate poisoning. It will be described in greater detail later. Its presence or absence has been recorded for all birds in the series.

Each experiment was continued until the functional condition of individual birds ceased to deteriorate. Selected birds were taken for histological examination and tissues were prepared as previously described (Cavanagh, 1954; Lancaster, 1960), using the Swank Davenport method for degenerating myelin and standard silver and dye methods for nervous tissues.

RESULTS

The neurotoxic effects of intramuscular tri-p-*ethylphenyl phosphate.* With doses of 500 to 1,000 mg/kg, ataxia and paralysis may occur at any time between 10 and 20 days, and in their manner of progression and final manifestations are virtually indistinguishable from those produced by dyflos or tri-*o*-cresyl phosphate.

The lowest dose causing disturbance of gait was 150 mg/kg. Above this and up to 500 mg/kg the onset of ataxia and weakness when they occurred was always delayed until 20 to 30 days and was nearly always preceded by a characteristic high-stepping gait. This began with an exaggerated but well-controlled upward

and forward movement of the leg which caused the foot frequently to brush the breast. The stride then continued with a rapid and powerful forward movement, the toes being fully extended as the movement progressed. Because the movement is a rapid one it can only be fully appreciated in a " slow-motion " film, but the general impression when actually observing the bird is of a long, graceful springy stride. In a few birds this type of gait disappeared only to reappear 5 to 10 days later, when it generally heralded ataxia during subsequent days (Table 1).

Occasionally with the onset of clumsiness a further striking but transient feature developed in which the high-stepping gait gave way to a stiff-legged stumbling run invariably ending after a few steps in the bird collapsing on its hocks. During this running phase the bird makes violent wing movements apparently to maintain its balance.

These features are no longer seen with the onset of ataxia and weakness, and the condition of the bird thus tends to merge into the state seen with higher doses. At threshold doses, individual birds not severely incapacitated (up to 4 points) may eventually show complete functional recovery.

*The oral toxicity of tri-*p*-ethylphenyl phosphate.* When tri-*p*-ethylphenyl phosphate was given orally all the characteristic features described above were seen. The onset and development of ataxia, as distinct from high-stepping gait, were, however, less variable and ultimately indistinguishable from those seen with tri-*o*-cresyl phosphate. High-stepping gait was always combined with some degree of ataxia and was never seen alone. Thus the controlled striding action seen after intramuscular dosing was replaced by a stiff-legged stumbling run that had also been seen occasionally in the injected birds during the transition period to the ataxic state.

With this method of dosing, ataxia developed after 8 to 10 days and its progress thereafter was rapid. The maximum effect was reached in a further 3 to 4 days, as is shown in Table 2.

*Structural changes in the nervous system of hens poisoned with tri-*p*-ethylphenyl phosphate.* Birds given 0.2 to 1.0 mg/kg either orally or intramuscularly were examined. In general, the overall distribution and pattern of neuronal damage were the same as those following dyflos or tri-*o*-cresyl phosphate (Cavanagh, 1954 ; Fenton, 1955). However, certain differences were recognized, but these were differences in emphasis rather than in the character of the lesions.

Thus the sciatic nerves were consistently and severely affected by tri-*p*-ethylphenyl phosphate. With high doses the degree of damage was indistinguishable from that due to tri-*o*-cresyl phosphate and dyflos. With lower doses the damage was more severe with tri-*p*-ethylphenyl phosphate than with low doses of either of the others. On the other hand, the ventral tract of the spinal cord was relatively little affected by tri-*p*-ethylphenyl phosphate. For instance, at the higher doses only about 12 degenerating and swollen axons were visible in a random section at the lumbar level, while at 0.5 mg/kg only one or two could be seen.

The damage to the spinocerebellar and ventral pathways was similar to that caused by tri-*o*-cresyl phosphate and dyflos, and no additional changes occurred in other tracts.

One difference between birds dosed orally and those dosed intramuscularly should be mentioned, since it confirms the conclusions discussed elsewhere concerning the effect of route of administration upon the manner of development of the damage. Orally dosed birds all showed changes that were in approximately the same stage of development at a given time, while intramuscularly injected birds, even a month later, showed axonal degeneration in an early stage side by side with more advanced changes such as gliosis. These findings imply a more gradual cumulative intoxication with the intramuscular route and are consistent with a slow release of toxic agent from a depot.

Comparison of the functional changes produced by intramuscular dyflos, tri-o-cresyl phosphate, and tri-p-ethylphenyl phosphate. To determine whether the features of tri-*p*-ethylphenyl phosphate poisoning described earlier were in fact unique to this substance, all three compounds were compared at various dose levels. These doses were chosen on the basis of previous experience so as to produce (*a*) severe, (*b*) moderate and (*c*) minimal effects. The birds were examined simultaneously. Table 1 shows that considerable variation in effect occurs both with the compound and with the dose.

TABLE 1

A COMPARISON OF THE INTRAMUSCULAR NEUROTOXICITY OF DYFLOS, TRI-*o*-CRESYL PHOSPHATE AND TRI-*p*-ETHYLPHENYL PHOSPHATE IN CHICKENS

Numbers indicate relative intensity of ataxia; – indicates absence of any signs of ataxia or paralysis; parentheses indicate the presence of high-stepping gait. All birds were given protective doses of oxime and atropine (see Methods)

	Dose mg/kg	Days after injection																	
		8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Dyflos	1·0	–	2	4	6	6	6	7	7	8	8								
			4	5	6	7	7	8											
	0·6	2	4		6														
		1	1	2	3	6	6	6	6	6	6								
	0·3				–	–	–	–	–	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
		–	–	–	2	–	2	2	2	–	2	1	3	2	3	3	3	2	2
		–	–	–	2	2	2	–	–	–	–	–	1	1	0	1	1	1	1
Tri-*o*-cresyl phosphate	400	–	–	–	2	4	6	8											
		–	–	–	2	4	6	8											
		–	–	(1)	(2)	4	4	4	5	5	5	7	7	7					
	150	–	–	–	–	–	–	–	2	2	2	3	4	6					
		–	–	–	–	1	1	1	3	4	5	5	6	5	6	6	6	6	
	75	–	–	(1)	–	4	5	6	6	7	7								
		–	–	–	–	(2)	(–)	(–)	(–)	(2)	(2)	2	3	4	5	5	5	6	
		–	–	–	–	–	–	–	–	3	4	4	5	6					
	50	–	–	–	–	–	–	–	1	1	1	1	–	2	1	–			
		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–			
		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–			
		–	–	–	–	–	–	–	2	2	2	5							
	30	–	–	–	–	–	–	–	1	–	–	1	1	1					
		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–			
		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–			
		–	–	–	–	–	–	–	–	–	2	2	–	2	1	1			
Tri-*p*-ethyl-phenyl phosphate	750	–	–	–	–	–	–	–	–	(–)	(–)	(–)	3	3	4	4	5	6	
		–	–	–	–	–	–	–	2	3	4	6	6	7					
		–	–	–	–	3	3	3	(4)	6	7								
	300	–	(–)	(–)	(–)	(–)	–	–	(–)	(–)	(–)	(–)	(1)	1	(–)	(–)	(2)	(2)	
		–	–	–	–	–	–	–	–	–	–	–	–	1	2	2	–	(1)	
		–	(–)	(–)	–	(–)	–	–	–	(–)	(–)	(–)	(–)	(2)	(3)	(4)	(4)	5	
	150	–	–	–	–	–	–	–	–	(–)	(–)	–	(–)	(1)	2	2	5	6	
		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
		–	–	–	–	–	–	–	–	–	–	(–)	(–)	(–)	(–)	(–)	(–)	(1)	(2)

With dyflos 6 to 8 points of dysfunction were obtained with doses of 1.0 and 0.6 mg/kg, but with 0.3 mg/kg only 2 out of 3 birds became ataxic, and these were but mildly affected. The third bird developed a high-stepping gait on the 16th day, which appeared to be very similar to, but not identical with, that seen in tri-*p*-ethyl-phenyl-phosphate-poisoned birds. In the latter, the high-stepping gait following intramuscular injection is characterized by a long, graceful springy stride. This is not so in the high-stepping gait following dyflos.

Tri-*o*-cresyl phosphate (400 mg/kg) produced severe effects in all birds (7 to 8 points). The severity of the effects was less at the lower doses (150 and 75 mg/kg). Three out of 16 birds (1 at 400 mg/kg and 2 at 75 mg/kg) showed a high-stepping gait, but, as with dyflos, the signs were not identical with those seen after tri-*p*-ethylphenyl phosphate.

The onset and rate of progress of neurotoxic signs were dose-dependent with all three compounds, although this was less obvious with dyflos. With this compound the delay in onset varied from 8 to 11 days, being longer delayed at the smallest dose tested. With tri-*o*-cresyl phosphate the first signs did not occur until 11 days after poisoning, and at threshold doses these were delayed for up to 17 days.

In contrast to this regular pattern the onset of signs in tri-*p*-ethylphenyl phosphate poisoning was most irregular. Thus even at 750 mg/kg only one bird showed signs of ataxia on the 12th day, whilst the remaining two showed no such signs until the 15th. At 300 to 150 mg/kg ataxia was not apparent until 19 to 24 days.

The route of administration influenced the neurotoxicity of these substances, as can be seen from Table 2. Thus dyflos was much less neurotoxic orally than intramuscularly. On the other hand, tri-*p*-ethylphenyl phosphate was more toxic orally and the effects came on earlier and were more consistent and more severe than when it was given by intramuscular injection.

TABLE 2

A COMPARISON OF THE ORAL NEUROTOXICITY OF DYFLOS AND TRI-*p*-ETHYL PHENYL PHOSPHATE IN CHICKENS

Numbers indicate relative intensity of ataxia; – indicates absence of any signs of ataxia or paralysis; parentheses indicate the presence of high-stepping gait. All birds were given protective doses of oxime and atropine (see Methods)

Compound	Dose mg/kg	Days after injection							
		7	8	9	10	11	12	13	14
Dyflos	0·6		–	–	–	–	–	–	–
		–	–	1	–	1	1	1	1
		–	–	1	–	–	–	–	–
	0·3	–	–	–	1	1	–	–	–
		–	–	–	–	–	–	–	–
		–	1	1	1	–	–	–	–
Tri-*p*-ethylphenyl phosphate	750		2	2	(5)	(5)	7	7	
		–	–	1		Died			
		–	(2)	(2)	(3)	(4)	(5)	7	
	300	–	–	(1)	4	4	5	6	
		–	–	1	(5)	(5)	7	7	
				1	4	5	5	6	

DISCUSSION

The observations reported here confirm those of Silver (1960) that the functional picture in tri-*p*-ethylphenyl phosphate poisoning shows characteristics which are not obvious in either dyflos or tri-*o*-cresyl phosphate poisoning. Thus the high-stepping gait which is such a feature after tri-*p*-ethylphenyl phosphate is seldom seen after the other two, although with them a similar, though not identical, sign is observed in occasional birds.

One of the most constant features in dyflos or tri-*o*-cresyl phosphate neurotoxicity is the interval to onset. With dyflos it is very regular and even at threshold doses only slightly prolonged. The delay period with tri-*o*-cresyl phosphate is rather more dose-dependent, but the pattern of onset is still quite marked. By contrast, the onset of ataxia in tri-*p*-ethylphenyl phosphate is very inconstant and markedly delayed.

These anomalous features of tri-*p*-ethylphenyl phosphate poisoning are influenced by the route of administration. Thus slow release from a depot following intramuscular injection has the effect of extending the time scale during which the train of symptoms develop. Oral dosing and increase of dose have, on the other hand, the effect of condensing the sequence of events and blurring the outlines of their components.

Even so, the characteristic high-stepping gait described above still dominates the picture whatever the route of administration. With the other two compounds, lowering the dose or giving it intramuscularly does not produce these characteristic signs, so that it does not seem unreasonable to agree with Silver (1960) that tri-*p*-ethylphenyl phosphate is not acting in the same manner on the nervous system as the other organophosphorus compounds.

The histological evidence gives further support to this conclusion. Reducing the dose of dyflos (Lancaster, 1960) and tri-*o*-cresyl phosphate (Cavanagh, unpublished results) reduces the extent of damage to the peripheral nerves to a greater degree that it does in the long tracts of the spinal cord. With tri-*p*-ethylphenyl phosphate the reverse would seem to hold, and furthermore there is a relative sparing of the ventral tracts. The effect is thus for this compound to produce a predominantly peripheral neuropathy at all dose levels.

The most compelling evidence in favour of the individuality of tri-*p*-ethylphenyl phosphate intoxication is its relative absence of anticholinesterase activity either *in vitro* or *in vivo* (Aldridge & Barnes, 1961). Up to the present time all the dialkyl phosphorofluoridates (Davies *et al.*, 1960) and all the triaryl phosphates (Hine, Dunlap, Rice, Coursey, Gross & Anderson, 1956) that produce the neurotoxic syndrome are anticholinesterases *in vivo*, although the converse does not hold.

This must therefore unequivocally set tri-*p*-ethylphenyl phosphate apart from the other neurotoxic organophosphorus compounds. Nevertheless the fact that in general the same neurone systems are attacked by tri-*p*-ethylphenyl phosphate as are damaged by the other organophosphorus compounds suggests that the final metabolic pathway disturbed by the former may well be the same as that disturbed by the latter. In view of its inertness towards cholinesterases, however, the intermediary

role of these enzymes as suggested for the alkyl phosphates by Davies *et al.* (1960) cannot be invoked. The interesting speculation thus suggests itself that the differing emphasis of the tri-*p*-ethylphenyl phosphate lesions may conceivably be an expression of its own as yet unknown intermediary metabolism. It is thus with much interest that further biochemical data about this substance are awaited.

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MULTIPLE TOE-PINCH METHOD FOR TESTING ANALGESIC DRUGS

BY

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In guinea-pigs and rats, an immediate squeak was one of the most consistent and readily observed responses to application of a light artery clip to the base of a toe. Morphine and related drugs suppressed this response. Squeak-responses from each toe of an experimental animal formed the basis of a technique for measuring activity of analgesic drugs. A statistical method was developed to analyse the correlated quantal observations obtained. It provided an estimate of the increase of information from several toes compared with one. Testing all toes of each animal yielded a substantial increase of information, because the correlation between responses of different toes was low. Among drugs having an analgesic action in man, 1-(β-diethylaminoethyl)-2-(*p*-ethoxybenzyl)-5-nitrobenzimidazole, methadone, morphine, pethidine and codeine (in descending order of potency) were active in this test in guinea-pigs. Acetylsalicylic acid, amidopyrine, amphetamine, chlorpromazine, 4-hydroxyisophthalic acid, lysergic acid diethylamide, mephenesin, nalorphine, pentetrazole, phenobarbitone, phencyclidine, phenytoin, salicylamide, strychnine and troxidone showed little or no activity. The time-courses of active drugs were estimated, and morphine had the longest action.

Haffner (1929) evaluated analgesic drugs by ability to suppress responses to a clip applied to the tail of a mouse or to the ear of a guinea-pig. Bianchi & Franceschini (1954) used a similar tail-pinch method in mice to investigate the potencies and time-courses of morphine, pethidine and methadone and to study the development of tolerance to these drugs.

We describe a method of measuring the activity of analgesic drugs in which a clip was applied, not to a single point, but to several comparable points on the same animal so as to increase the information yielded by each animal. The correlated quantal observations obtained required a novel statistical analysis. We have used mainly guinea-pigs to estimate the potencies and time-courses of some known analgesic drugs, testing all toes of each animal. Experiments with rats have shown that the multiple toe-pinch method can be used with a second toed species, and it seems suitable for others.

Warner & Collier (1960) have communicated an account of the statistical procedure involved in the multiple toe-pinch test. The pharmacological findings were reported to the British Pharmacological Society in January, 1961.

METHODS

Pharmacological methods

Male albino guinea-pigs, about 4 weeks old and ranging in weight from 150 to 300 g, were used, each animal being submitted to experiment once only. During experiments, animals were caged singly but within sight of one another. Responses were tested with an artery clip of light tension, the jaws of which were sheathed in polythene tubing. It was applied in random sequence to the bases of the 14 toes and, in untreated animals only, to both ears and to 4 places on the loose skin of the back (Fig. 1). In most experiments with analgesic

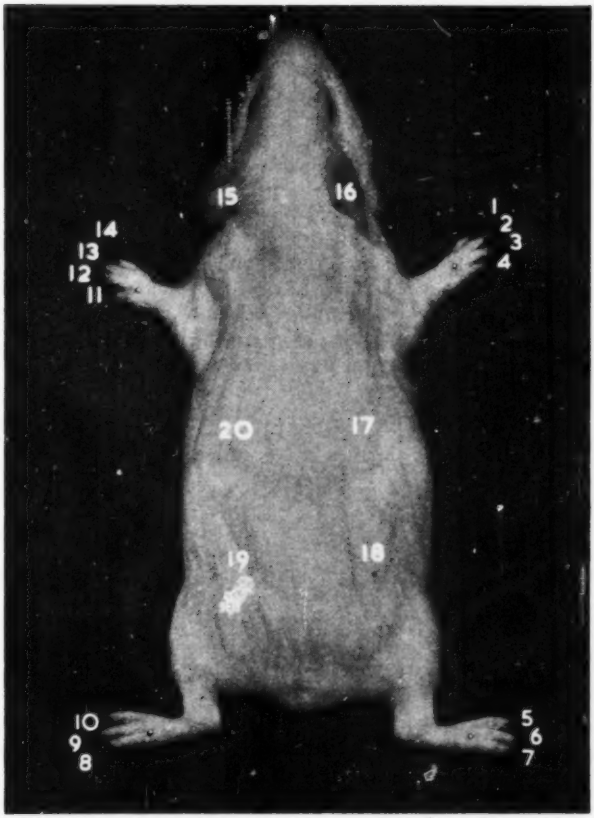

Fig. 1. Guinea-pig, showing points of application of the artery clip.

drugs the clip was applied once to each of the toes before administration of drug and at predetermined times afterwards, each round of 14 applications making one cycle of testing. In a few experiments a cycle consisted of 14 applications to toe 2 (see Fig. 1) at intervals of 5 sec. The clip was removed as soon as it was clear whether an immediate squeak (the squeak-response) was forthcoming, usually within 2 to 3 sec. Some experiments were performed with white rats, about 3 weeks old and weighing about 50 g.

Substances tested. Acetylsalicylic acid and its calcium salt, prepared by mixing 10 parts by weight of the acid with 3 of calcium carbonate and 1 of citric acid and dissolving in 0.9%

sodium chloride solution immediately before use; amidopyrine; amphetamine sulphate; chlorpromazine hydrochloride; codeine phosphate; 1-(β-diethylaminoethyl)-2-(*p*-ethoxybenzyl)-5-nitrobenzimidazole; 4-hydroxyisophthalic acid; lysergic acid diethylamide bitartrate; mephenesin; methadone hydrochloride; morphine sulphate; nalorphine hydrobromide; pentetrazole; pethidine hydrochloride; phenobarbitone sodium; 1-(1-phenylcyclohexyl) piperidine hydrochloride (phencyclidine; Chen & Weston, 1960); phenytoin sodium; salicylamide; strychnine sulphate; troxidone. Doses are expressed throughout as weights of active acid or base.

Statistical methods

Computations were based on the score which was taken as the proportion of applications of the clip which failed to elicit a squeak in one animal in one cycle of testing. The mean scores in dosage groups were subjected to the angular transformation (Finney, 1952b) and regression lines against log dose fitted by the usual iterative procedure, so that for any compound the ED50 (the dose for which the mean score was 0.50) could be estimated. To compare compounds, parallel lines were fitted at the times of maximal effect for each, and the ED50 values and relative potencies determined from these lines. To investigate the time-course of action of a drug, lines were fitted independently at each time at which observations were made. The 95% fiducial limits of the ED50 values and relative potencies were determined using Fieller's Theorem (Fieller, 1940) after estimation of the residual standard deviation.

Estimation of residual standard deviation. The computations differed from the usual quantal procedure in that the residual standard deviation had to be calculated from the results instead of being taken as unity. Suppose there are k dosage groups of n pigs, the score on the jth pig of the ith group is p_{ij}, and the mean score for the ith group is $\bar{p}_i$. Suppose also that the fitted angle for the ith group at the final stage of the iterative procedure is Y_i, corresponding to a probability of a positive response of P_i. The angular transformation is

$$P_i = \sin^2 Y_i$$

The residual sum of squares to be used in this analysis is

$$\sum_{i=1}^{k} \frac{\sum_{j=1}^{n} (p_{ij} - \bar{p}_i)^2}{P_i\,(1-P_i)} = 4 \sum_{i=1}^{k} \frac{\sum_{j=1}^{n} (p_{ij} - \bar{p}_i)^2}{\sin^2 2Y_i}$$

and this has $k(n-1)$ degrees of freedom. The left-hand side of the equation shows that the residual is a sum of ratios of the observed variance between scores in the same dosage group to the binomial variance that would be expected if only one quantal variate/guinea-pig was observed. The right-hand side was used in computation.

The computational procedure is illustrated in Table 1, using results of the experiment of Fig. 2. The notation is that of Finney (1952a, b). The first fitted line is obtained from empirical angles corresponding to the mean scores, $\bar{p}_i$. Two cycles of iteration are necessary to get successive values of Y_i differing by less than 1°, and the values of y_i and Y_i in the second cycle were used subsequently. The sums of squares and products, S_{xx}, S_{xy}, S_{yy}, were calculated giving a weight of one to each dose, so that the numerical factor appearing in the analysis of variance table is

$$5w = 5 \times \frac{4\pi^2}{(180)^2} = 0.0060925$$

where the 5 occurs because there are 5 pigs in each dosage group.

Information ratio. An analysis of 5 independent quantal responses at each dose would be the same as above except that the residual mean square would be unity. It follows that the reciprocal of the residual mean square is an estimate of the ratio of information obtained from several quantal observations to that expected from one. We have called this the information ratio, and, for the example in Table 1, it is 2.94.

TABLE 1

COMPUTATIONAL PROCEDURE FOR ESTIMATING ED50 VALUES, RESIDUAL STANDARD DEVIATION AND INFORMATION RATIO

Results were obtained in one experiment in which guinea-pigs were tested 1 hr after receiving morphine subcutaneously. The clip was applied to 14 toes and the score is the proportion of applications that failed to elicit a squeak. The information ratio is the ratio of information obtained using more than one toe to that expected from one

Group i	Dose of morphine (mg/kg)	x_i	Score for each guinea-pig p_{ij}	Mean score $\bar{p}_i$	Empirical angle	Y_i	1st cycle y_i	1st cycle Y_i	2nd cycle y_i	2nd cycle Y_i	$\sin^2 2Y_i$	$\Sigma(p_{ij}-\bar{p}_i)^2$
1	2·825	−2	0 0·36, 0 0 0	0·07	15·3	12·8	15·6	13·8	15·4	13·9	0·2175	0·1020
2	4·0	−1	0·57, 0·14, 0 0·43, 0·14	0·26	30·7	30·2	30·7	30·2	30·7	30·1	0·7531	0·2204
3	5·65	0	0·43, 0·07, 1·00, 0·71, 0·43	0·53	46·7	47·5	46·7	46·6	46·7	46·4	0·9976	0·4857
4	8·0	+1	0·86, 0·29, 0·57, 0·64, 1·00	0·67	54·9	64·9	53·7	63·0	54·2	62·6	0·6677	0·3020
5	11·3	+2	1·00, 1·00, 1·00, 1·00, 1·00	1·00	90·0	82·2	86·1	79·4	84·8	78·8	0·1452	0

$S_{xx}=10$, $S_{xy}=162{\cdot}3$, $S_{yy}=2742{\cdot}972$, $\bar{x}=0$, $\bar{y}=46{\cdot}36$, $b=S_{xy}/S_{xx}=16{\cdot}23$

Fitted line, $Y=y+b(x-\bar{x})=46{\cdot}36+16{\cdot}23\,x$

When $Y=45$, $x=-0{\cdot}0838$.

Hence ED50 $=5{\cdot}65\ \text{antilog}_{10}(-0{\cdot}0838\ \log_{10}\sqrt{2})=5{\cdot}5$ mg/kg.

Nature of variation	Sum of squares	d.f.	Mean square	Variance ratio
Regression	$0{\cdot}0060925\ (S^2_{xy}/S_{xx})=16{\cdot}048$	1	16·048	47·2, $P \ll 0{\cdot}001$
Means about regression	$0{\cdot}0060925\left(S_{yy}-\frac{S^2_{xy}}{S_{xx}}\right)=0{\cdot}663$	3	0·221	<1, not significant
Residual	$4\sum_i \frac{\Sigma(p_{ij}-\bar{p}_i)^2}{\sin^2 2Y_i}=6{\cdot}808$	20	0·340	

95% fiducial interval for ED50 = 4·7–6·3 mg/kg. Information ratio $=(0{\cdot}340)^{-1}=2{\cdot}94$.

RESULTS

Responses of untreated guinea-pigs to the clip

A guinea-pig responds to external application of an artery clip in a number of ways that depend partly on the tension of the clip and on the site of application. Responses include squeaking, starting, flattening the ears, opening and closing the mouth, shaking or withdrawing the limb if the clip is applied to a toe and struggling. The squeak-response was chosen for experiments with analgesic drugs, since it occurred almost immediately and was readily observed as a quantal effect.

Table 2 gives the squeak-responses obtained from 3 untreated guinea-pigs when the artery clip was applied at 0, 30 and 60 min in random order to the 20 points illustrated in Fig. 1. This table shows that guinea-pigs nearly always responded

TABLE 2

SQUEAK-RESPONSES OF UNTREATED GUINEA-PIGS TO REPEATED APPLICATIONS OF AN ARTERY CLIP AT HALF-HOURLY INTERVALS

The points of application are shown in Fig. 1

Time of test (min)	Guinea-pig no.	No. of squeaks		
		14 toes	2 ears	4 skin points
0	1	14	1	0
	2	13	0	0
	3	11	0	1
30	1	14	1	0
	2	14	0	1
	3	13	0	1
60	1	14	1	1
	2	14	0	2
	3	14	0	1

to applications of the clip to their toes, but not to their ears or skin. Although a clip of greater tension might have evoked a consistent response from ears or skin, we used the toes because a light clip seemed less likely to damage tissues and because the toes presented multiple similar points for application.

Table 2 also shows that the proportion of squeak-responses increased with repeated applications of the clip to toes or skin. Since its second application to the toes elicited almost 100% squeak-responses, the clip was applied to every toe immediately before drug treatment.

Responses of treated guinea-pigs to the clip

Morphine in sufficient dose suppressed squeak-responses to all applications of the clip to the toes. Fig. 2 shows examples of dose-response lines obtained on different occasions with morphine by three different routes. The values plotted are those at the times of peak effect.

Table 3 gives the average values of the ED50, slope and information ratio, and the coefficient of variation between the ED50 values, obtained from 25 experiments in which morphine was given by one of three routes. The subcutaneous route was chosen for general use, because the average slope and information ratio were greatest and variation between estimates of the ED50 value least.

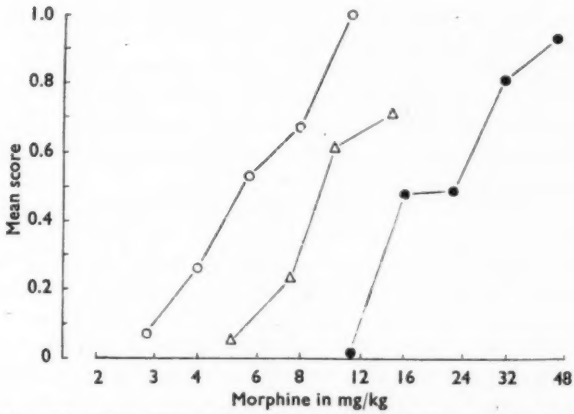

Fig. 2. Examples of log dose-response lines for morphine by three routes in guinea-pigs. For definition of mean score, see Table 1. ○ — ○ subcutaneous, 1 hr after treatment, 5 animals/dose. △— △ intraperitoneal, 1 hr after treatment, 4 animals/dose. ● — ● oral, 4 hr after treatment, 6 animals/dose.

TABLE 3

AVERAGE VALUES OF THE ED50, SLOPE AND INFORMATION RATIO, AND COEFFICIENTS OF VARIATION BETWEEN THE ED50 VALUES, OBTAINED IN 25 EXPERIMENTS WITH MORPHINE BY THREE ROUTES IN GUINEA-PIGS

For explanation of information ratio, see Table 1

Route of administration	No. of tests	Total no. of guinea-pigs	Average ED50 (mg/kg)	Average slope (°/log dose)	Average information ratio	Coefficient of variation between ED50 values (%)
Intraperitoneal	13	156	13·8	67	1·92	37
Oral	4	76	11·1	59	1·95	66
Subcutaneous	8	102	6·3	82	4·13	26

By this method, four morphine-like drugs—codeine, pethidine, methadone and 1-(β-diethylaminoethyl)-2-(*p*-ethoxybenzyl)-5-nitrobenzimidazole — gave dose-response lines from which potencies were estimated (Table 4). Consistent results were obtained in two independent experiments in each of which codeine and

TABLE 4

SOME ANALGESIC DRUGS IN GUINEA-PIGS. POTENCIES RELATIVE TO MORPHINE AND INFORMATION RATIOS

Relative potencies were determined from minimal values of ED50 obtained in the same experiment using the same route of administration. s.c.=subcutaneous; i.p.=intraperitoneal. 20684-Ba is 1-(β-diethylaminoethyl)-2-(*p*-ethoxybenzyl)-5-nitrobenzimidazole. For explanation of information ratio, see Table 1

Drug	Route of administration	Potency relative to morphine (95% fiducial interval)	Information ratio
Codeine	s.c.	0·19 (0·13–0·25)	5·6
	s.c.	0·23 (0·19–0·27)	2·7
	i.p.	0·38 (0·27–0·63)	2·7
Pethidine	s.c.	0·25 (0·18–0·35)	6·3
	s.c.	0·37 (0·31–0·45)	13·4
	i.p.	0·46 (0·30–0·74)	1·6
Methadone	s.c.	2·5 (1·9–3·3)	2·3
20684-Ba	s.c.	1,960 (1,650–2,460)	4·9

pethidine were given subcutaneously. The information ratios in Table 4 range between 1.6 and 13.4, showing that, with each drug, testing 14 toes yielded considerably more information than would have been obtained by testing only one.

Some drugs not of the morphine type but having or reported to have analgesic effects were also tested. Given subcutaneously, acetylsalicylic acid (800 mg/kg) and its calcium salt (400 mg/kg), amidopyrine (160 mg/kg), amphetamine (50 mg/kg), chlorpromazine (64 mg/kg), 4-hydroxyisophthalic acid (400 mg/kg), lysergic acid diethylamide (1 mg/kg), nalorphine (128 mg/kg), phencyclidine (16 mg/kg) and salicylamide (400 mg/kg) did not suppress the squeak-response. Although itself ineffective, nalorphine (32 mg/kg) antagonized the effect of morphine (8 mg/kg) given half an hour afterwards, both drugs being administered subcutaneously.

Some anticonvulsant drugs were given subcutaneously at doses which retarded but did not abolish the righting reflex. Of these, mephenesin (800 mg/kg), phenobarbitone (50 mg/kg) and troxidone (1 g/kg) did not affect the squeak-responses; but phenytoin (50 mg/kg) suppressed about one-third of these responses.

The convulsant drugs pentetrazole (50 mg/kg) and strychnine (2 mg/kg), when given subcutaneously in amounts just below the lethal dose, failed to suppress the squeak-response.

TABLE 5

TIME-COURSES OF SOME ANALGESIC DRUGS IN GUINEA-PIGS

s.c.=subcutaneous; i.p.=intraperitoneal; p.o.=oral. 20684-Ba is 1-(β-diethylaminoethyl)-2-(*p*-ethoxybenzyl)-5-nitrobenzimidazole

Drug	Route of administration	No. of guinea-pigs	Time after administration in hr	ED50 (95% fiducial interval) in mg/kg
Morphine	s.c.	20	0·5	6·5 (4·7–8·0)
			1	5·4 (4·4–6·6)
			2	5·1 (4·1–6·4)
			4	6·0 (4·7–7·5)
			6	11·8 (8·6–18·7)
Morphine	p.o.	30	0·5	36·7 (29·3–55·9)
			1	26·0 (18·9–42·2)
			2	22·3 (15·8–29·8)
			4	21·4 (16·9–26·4)
			6	28·6 (23·5–39·0)
Codeine	s.c.	15	0·25	25·8 (17·9–32·6)
			0·5	25·7 (18·8–31·6)
			1	40·4 (27·4–826)
			2	>50
Pethidine	s.c.	15	0·25	15·9 (13·6–18·0)
			0·5	16·1 (14·7–17·6)
			1	22·2 (18·8–28·2)
			2	⩾30
Pethidine	i.p.	20	0·25	26·4 (14·4–31·9)
			0·5	30·2 (22·2–39·8)
			1	46·0 (36·7–68·1)
			2	>64
Methadone	s.c.	12	0·25	3·6 (2·8–6·3)
			0·5	3·2 (2·5–4·7)
			1	>4·5
20684-Ba	s.c.	15	0·25	0·0037 (0·0018–0·0059)
			0·5	0·0037 (0·0028–0·0059)
			1	0·0049 (0·0034–0·0066)
			2	>0·0083

TABLE 6

NUMBERS OF SQUEAK-RESPONSES CLASSIFIED BY ANATOMICAL POSITION OF TOES AND BY TEMPORAL ORDER OF TESTING IN SIX EXPERIMENTS WITH ANALGESIC DRUGS IN GUINEA-PIGS

The positions of toes are shown in Fig. 1. Toes were tested in random sequence. Responses, at the observed time of peak effect, at all dose-levels within each experiment are pooled

		Total squeak-responses																									
		By anatomical position														By temporal order of testing													
Drug	No. of pigs	1	2	3	4	5	6	7	8	9	10	11	12	13	14	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Morphine	24	12	16	14	13	14	17	14	12	15	12	6	13	14	8	9	9	10	12	11	13	13	13	14	13	17	17	15	14
Morphine	18	9	10	6	8	7	7	11	6	9	11	6	5	9	10	3	5	7	7	7	10	9	8	8	7	9	11	11	12
Codeine	15	5	6	4	6	7	8	8	7	7	8	3	5	5	4	2	4	5	5	6	7	6	6	6	8	8	8	5	7
Pethidine	15	4	5	7	7	6	8	8	7	6	7	4	6	7	8	2	3	5	6	6	9	6	8	7	7	6	7	10	8
Morphine	18	10	6	8	6	8	6	7	6	4	6	4	3	8	6	2	1	6	4	5	6	7	6	7	9	8	10	7	10
Morphine	25	12	13	13	11	14	13	15	13	16	10	5	12	14	12	7	10	9	10	10	9	11	13	17	15	16	15	15	16
All	115	52	56	52	51	56	59	63	51	57	54	28	44	57	48	25	32	42	44	45	54	52	54	59	59	64	68	63	67

Time-courses of actions. The time-courses of active drugs were studied by testing the same animals at a number of times after treatment. Table 5 gives the time-courses of 5 drugs in terms of the ED50 values obtained at each time of testing. Of the drugs examined, morphine was by far the longest-acting, for its effect did not lessen appreciably until >4 hr after treatment by oral or subcutaneous routes.

Position and order of testing of toes. To examine how the anatomical position of toes and temporal order of testing affected the response, we analysed results from a number of experiments with analgesic drugs. The numbers of squeak-responses obtained in 6 experiments are classified by position and order in Table 6. Analysis showed that the numbers of responses were significantly different with respect both to anatomical position ($P<0.001$) and temporal order ($P<0.00001$). By an approximate test for outliers (Tukey, 1949), the toe in position 11 was significantly ($P<0.00001$) less responsive than the remaining 13 toes, which did not differ significantly ($P>0.20$) from one another. Although the differences were not significant, the two next least responsive toes (12 and 14) also occurred on the left forelimb.

In all experiments in Table 6, the animal was held with the left hand and the clip applied to its toes with the right. To investigate whether the unresponsiveness of toe 11 was due to a bias inherent in this way of handling, such as a difference in accessibility, two experiments were performed, in which the operator reversed the actions of left and right hands. In these, toe 11 was slightly more responsive than average, while toe 4, the corresponding toe on the opposite side, was slightly less responsive than average.

Table 6 also shows that, as the cycle of testing proceeded, responsiveness increased. Plotting total squeak-responses in Table 6 against order of testing gave a line that had significant slope ($P\ll0.00001$) and did not deviate from linearity significantly ($0.1<P<0.25$). In all experiments in this table, the 14 toes were tested in random sequence as quickly as practicable, the cycle taking about 1 min. To explore how time between testing of individual toes affected responsiveness, we carried out 2 experiments, in which toes were tested 1 hr after treatment with morphine, at intervals of 30 sec in one experiment and of 2 min in the other. In both of these, the proportion of squeak-responses increased with temporal order of testing.

TABLE 7

CORRELATIONS BETWEEN TOES BY ANATOMICAL POSITION IN TWO INDEPENDENT EXPERIMENTS IN GUINEA-PIGS

Average correlation between pairs of toes	No. of correlations	Expt. 1 (48 animals)	Expt. 2 (18 animals)
All possible	91	0·21	0·32
On different feet	73	0·20	0·31
On same foot	18	0·24	0·35
Adjacent on same foot	10	0·29	0·39
Non-adjacent on same foot	8	0·18	0·29
Corresponding on left and right feet	7	0·20	0·20

The product moment correlation coefficients between responses from pairs of toes, classified by anatomical position and temporal order of testing, were computed. The results for anatomical position from two experiments involving 48 and 18 guinea-pigs are summarized in Table 7. The average correlations between all

possible pairs of toes were low. As might be expected, average correlations between pairs of toes both on the same foot were higher than those between pairs on different feet and average correlations between adjacent toes were higher still, though all differences were small. The average correlations between corresponding toes on left and right sides were slightly lower than the overall average. The significance of these differences could not be tested. When toes were classified by temporal order of testing, correlations between pairs did not seem to depend on the number of intervening toes.

Information from different numbers of toes. In all experiments with guinea-pigs reported above, 14 toes were tested. By analysing separately results from selected toes, we have studied the relationship between number of toes tested and information gained. Information ratios were determined for even numbers of toes, chosen either by anatomical position or by temporal order. With either classification, comparable results were obtained. For example, using results from the larger experiment in

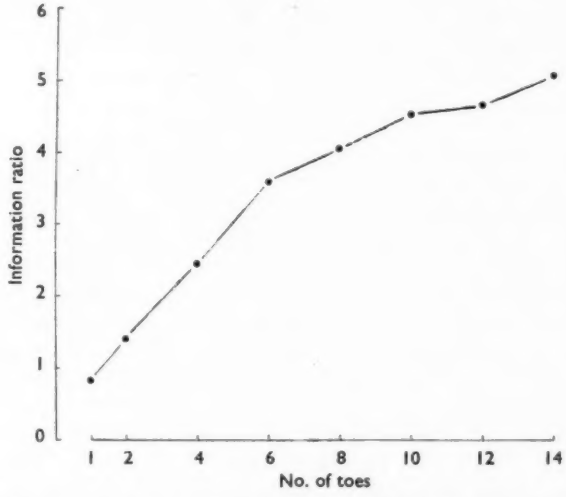

Fig. 3. Increase of information with number of toes. To calculate information ratios (for explanation, see Table 1), toes were taken in temporal order of testing. The results are from a comparison of morphine, pethidine, and codeine by the subcutaneous route in an experiment using 48 guinea-pigs.

Table 7, information ratios were calculated for different numbers of toes taken in temporal order. These are plotted in Fig. 3, which shows, as might be expected, that information increased with the number of toes tested, but the rate of increase declined.

Although increasing responsiveness with temporal order of testing did not change the shape of the curve in Fig. 3, it caused a rise in ED50 values as more toes were included. For example, the ED50 of morphine for the first 6 toes tested was 4.5 and for all toes 5.9 mg/kg.

Repeated testing of a single toe. In two experiments with morphine, a clip was applied 14 times to toe 2, and, in one reference experiment, the same clip was applied as usual once to each toe per test cycle. The results of these experiments are given in Table 8, which shows that morphine was considerably more potent when

TABLE 8

REPEATED TESTING OF A SINGLE TOE. ESTIMATES OF ED50, SLOPE AND INFORMATION RATIO 1 HR AFTER MORPHINE SUBCUTANEOUSLY

For explanation of information ratio, see Table 1

Form of test	Slope in °/log dose	ED50 (95% fiducial interval) in mg/kg	Information ratio
14 toes tested once	89	7·2 (5·5–9·2)	3·0
1 toe tested 14 times	28	1·2 (0·4–2·1)	2·1
	43	1·6 (0·7–3·6)	1·8

one toe was tested repeatedly, but the slope of the dose-response curve and the information ratio were lower. Another difference from our findings with the usual procedure was that the increase of responsiveness of the one toe to the clip was not evident as the cycle proceeded.

Experiments with rats

Preliminary experiments showed that weanling white rats squeaked sharply when the clip was applied to the base of a toe. In one experiment, morphine was administered subcutaneously at different doses to 4 groups of animals and saline to a fifth group. The clip was applied in random sequence to the 18 toes of each animal before treatment and 30, 60 and 120 min after treatment. Morphine at 10 mg/kg completely, and at lower doses partially, suppressed the squeak-responses. The peak ED50 value at 30 min was estimated as 3.3 mg/kg, with a 95% fiducial interval of 2.8 to 3.9 mg/kg, and the information ratio was 5.5. After the lower doses of morphine, the toes on the forelimbs were on average less sensitive than those on the hindlimbs.

DISCUSSION

The term " analgesic " has two disadvantages. First, depression of pain may not be complete, so that the effect would be better described as " hypoalgesic." However, the term analgesic is so well established that we have not attempted to introduce a new one. The second disadvantage applies to animal tests, in which we do not know whether the subject actually feels pain and where, therefore, both terms analgesic and hypoalgesic may be inappropriate. Winder (1959) has applied the term " nociceptive " to reactions to noxious stimuli in animals and " antinociceptive " to drugs reducing such reactions. In the present work the compounds found active are known to relieve pain in man, and for these we have therefore continued to use the term analgesic drug.

Of the drugs examined, diethylaminoethylethoxybenzylnitrobenzimidazole, methadone, morphine, pethidine and codeine, in descending order of potency, were capable of suppressing the squeak-response completely. They suppressed not only the

squeak-response but other responses to the clip, and their order of potency corresponded with that in man. Substances of other types that relieve pain in man, such as antipyretic drugs, did not show appreciable activity in the multiple toe-pinch test in guinea-pigs. The failure of mephenesin, phenobarbitone and troxidone and of pentetrazole and strychnine to suppress the squeak-response showed that this response was insensitive to a number of non-analgesic drugs which are known to have central actions. The foregoing evidence suggests that the multiple toe-pinch test in guinea-pigs measures analgesic activity of the morphine type.

The method of testing one toe 14 times does not commend itself because, with morphine, the slope of the log dose-response curve and the information ratio were lower and the fiducial interval of the ED50 value was wider. However, as the potency of morphine was much greater, this variation of method might be useful for detecting the action of weak analgesic drugs.

The multiple toe-pinch test was successfully used in rats as well as guinea-pigs. Chicks also give a clear response to application of a clip to a toe (Schneider, 1961). There therefore seems good reason to think that this method of examining analgesic drugs can be used in a variety of toed species. We can envisage its extension to other sites or forms of stimulation. The principle of making correlated quantal observations so as to increase information from each experimental unit seems capable of wider application.

In the usual routine of testing, the posterior toe of the left forelimb was less responsive than the others. When the operations of the hands of the experimenter were exchanged, this lack of responsiveness disappeared, but was observed in the corresponding toe of the right forelimb. This bias arose from the right- or left-handedness of the operator.

The possibility that responsiveness to the clip may be greater in later cycles of testing suggests that experiments on the time-courses of analgesic drugs should be interpreted with caution. With morphine, peak potency was reached from 1 to 4 hr after treatment and after one or more cycles of testing. In this instance, at least, increase of responsiveness with testing has been more than compensated by increase in drug effect.

The lower responsiveness of one toe (no. 11) and the increasing responsiveness with temporal order of testing were seen with every active drug examined. These effects therefore seem unlikely to invalidate comparisons of potencies of drugs.

The increase in responsiveness during tests in which the clip was applied to all toes might be attributed to conditioning. Two facts, however, seem to contradict this explanation. First, responsiveness did not increase when only one toe was tested 14 times in a cycle. Secondly, the animals developed no signs of fear or distress in response to circumstances associated with application of the clip.

The information ratio is a novel feature of the statistical method. If all toes of an animal reacted alike, there would be no advantage in testing more than one toe and the information ratio would be theoretically unity. If the responses were not correlated, the information ratio would be equal to the number of toes tested. As the correlation between the responses decreased, the information ratio rose. For

example, in the experiments of Table 7, in which the average correlations between pairs of toes were 0.32 and 0.21, the information ratios were 3.5 and 4.7 respectively.

With guinea-pigs, it would be possible to test any number of toes up to 14 on each animal. Fig. 3 suggests that the information ratio would increase as the number of toes tested increased. For ethical reasons and for economy of animals, all the toes were tested in order to obtain the maximum information from each animal. When all toes are tested the information ratio exceeds unity and this expresses the main economic advantage of the test.

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THE EFFECT OF ATROPINE, PROPANTHELINE AND POLDINE ON THE VAGALLY STIMULATED GASTRIC MOTILITY AND THE HISTAMINE-STIMULATED ACID GASTRIC SECRETION IN THE RAT

BY

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Histamine-induced acid gastric secretion in the anaesthetized rat was not diminished by poldine in a dose which reduced vagally stimulated gastric contractions by approximately 75%. A dose of atropine, twice as large as the dose which reduced gastric contractions by 75%, had no apparent effect on the histamine-stimulated acid gastric secretion up to 2 hr after the injection. Only when more than 40 times as much atropine was injected did a slight inhibition of the acid secretion occur in 80 to 120 min. Propantheline, in a dose which inhibited gastric contractions by approximately 75%, slightly diminished acid secretion in 40 to 80 min. This effect was not increased by a further dose of propantheline. It was concluded that, in so far as any inhibition of acid gastric secretion had occurred, this could not be interpreted as an anti-muscarine or a direct toxic effect, but rather as an indirect effect possibly due to interference with the blood flow through the stomach wall.

Atropine, propantheline and poldine are used clinically to depress gastric secretion. Their effect is generally explained in terms of blockade of the muscarine effect of locally released acetylcholine. In high dosage these compounds have a transient ganglion-blocking action (Bainbridge & Brown, 1960); they may also be feebly antihistaminic (Acred, Atkins, Bainbridge, Brown, Quinton & Turner, 1957). Nevertheless it is unlikely that their apparent clinical usefulness in depressing gastric secretion depends on these effects: more powerful antihistamines such as mepyramine do not depress gastric secretion. Janowitz & Hollander (1956) found in the dog that atropine sulphate in doses of 0.2 and 0.4 mg/kg reduced the rate of secretion in vagally denervated gastric pouches which were stimulated by histamine: the acidity, however, remained relatively constant and independent of the secretory rate. They also showed that inhibition increased with increasing doses of atropine and also with time, being greatest in the third hour after its injection. Although atropine sulphate in a dose of 0.4 mg/kg produced toxic effects, the authors were unable to explain the effect of this dose on secretion solely as a toxic manifestation of the drug, since inhibition developed gradually. The same slow onset of inhibition occurred even with the lowest dose.

In previous experiments with rats we have shown that phloxin acts rapidly to inhibit acid gastric secretion (van Noordwijk & Aarsen, 1954). In contrast with atropine, it had no effect on gastric motility or peptic activity (Aarsen, 1959). We

were therefore interested to study the effect on the histamine-stimulated acid gastric secretion in this species of atropine and atropine-like drugs applied in doses which reduced or abolished the motility of the stomach. The required doses were found by recording the effect of these drugs on gastric contractions induced by vagal stimulation.

METHODS

Female rats of 200 to 250 g were used in all experiments. The animals were starved during the preceding night, and were anaesthetized by a single intramuscular injection of 0.6 ml./100 g of 25% solution of urethane.

Operative technique. This was the same for recording the gastric contractions and the acid secretion, except that for the former the right vagus nerve in the neck was exposed, ligated and cut so that the peripheral end could be stimulated. The trachea and the left jugular vein were exposed and cannulated. The abdomen was opened through a transverse incision just below the costal arch ; both epigastric arteries were ligated. Then the oesophagus was exposed at the cardia and the ventral vagus trunk lying on the oesophagus was dissected. A polythene tube of 14 cm length and 2 mm external diameter was passed into the stomach via the oesophagus and fixed at the cardia excluding the vagus trunk. A polythene cannula of 5 mm external diameter and bevelled at the tip was introduced into the stomach through an incision in the duodenum and fixed by a ligature round the pylorus. Directly behind the bevelled tip this cannula had two lateral holes to prevent recess formation around this end protruding in the antrum. Before closing the abdominal wall the stomach was rinsed by perfusing it swiftly with pre-warmed physiological saline (N/4,000 sodium hydroxide was used in the experiments for recording acid gastric secretion). At the same time gentle massage was applied to the stomach.

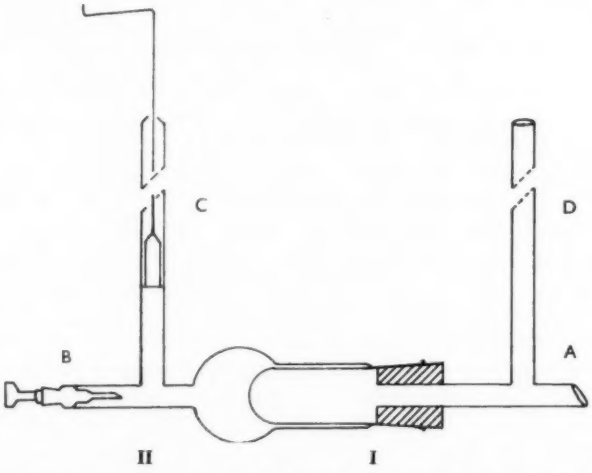

Fig. 1. Modified tambour used for recording of gastric contractions in the rat. The gastric cannula was attached to side-arm A of the compartment I. The compartments I and II were separated from each other by a thin rubber membrane (finger cot). Compartment I was filled with physiological saline; compartment II with a saturated solution of sodium sulphate. The volume in this second compartment could be changed through the side-arm B. The vertical glass tube D served as a manometer. A thin glass rod bent at the tip served as a writer. This was attached to the light polythene float in the glass tube C.

Recording of gastric contractions. These were recorded with a modified tambour (Fig. 1). The part connected to the gastric cannula (A) was completely filled with physiological saline at a pressure of 8 cm of water. The part which included a thin rubber membrane (finger cot) was filled with a saturated solution of sodium sulphate. Linear recording of pressures in the range from 4 to 15 cm of water could be obtained by changing the vol. of sodium sulphate in the second compartment through the side arm (B). A thin glass rod bent at the tip was connected to the light polythene float in the vertical glass tube (C), and served as a writer. The vertical glass tube (D) served as a manometer; the stomach was adjusted to the zero level of this manometer. At the beginning of the experiment the pressure in the compartment (I) connected to the saline-filled stomach was adjusted to 8 cm of water by adding saline via the manometer (D). The peripheral end of the cut right vagus nerve was stimulated by stainless steel electrodes applied to the nerve only during the periods of stimulation. The electrodes were connected to an electronic stimulator via an interruptor so that the nerve was stimulated for 5 sec periods, at 15 sec intervals. The square wave pulses had a duration of 5 msec at a frequency of 10/sec. The current was adjusted until the contractions became regular for this frequency and duration of stimulation; in most experiments the current was about 0.04 mA.

Recording of acid gastric secretion. The method of Ghosh & Schild (1958) for the continuous recording of the total acid secretion was used. However, we did not open the stomach to remove food debris. This method is based on continuous perfusion of the stomach with N/4,000 sodium hydroxide and recording of the *p*H of the effluent. This method makes it possible to test the effect of several successive intravenous doses of histamine in the course of one experiment lasting several hr. Repeated administration of the same dose of histamine usually produces constant effects. In nearly all experiments the constant dose of histamine base was 100 μg. For some rats, however, this dose was too large or too small; in these animals we used 50 or 200 μg of histamine for consecutive stimulation of the acid gastric secretion. All intravenous injections of histamine, except the first, were given when the gastric effluent had reached *p*H 6. The responses are expressed in terms of the lowest *p*H value measured after the injection of the drug.

Anti-acetylcholine action in vitro. A 6 to 7 cm segment of terminal ileum of guinea-pigs weighing 250 to 300 g was suspended in 15 ml. oxygenated Tyrode's solution from which magnesium chloride had been omitted. The temperature was kept at 35° C. A dose cycle of 2 min with 10 sec contact was used. The experiments were carried out as described by Schild (1947). Three doses of acetylcholine alone and three double doses of acetylcholine in the presence of a constant dose of antagonist were administered in random order. This series was repeated 4 times on each preparation. The antagonist was added 60 sec before acetylcholine. Regression-lines were fitted to the results of these assays according to the method of least squares (Emmens, 1948).

The following salts of the 3 drugs were used: atropine sulphate, propantheline bromide (Pro-Banthine) and poldine methylmethosulphate (Nactate). The doses given below are expressed in terms of the salts.

RESULTS

Effect on gastric contractions. Two experiments were performed in which the vagus nerve was stimulated repeatedly as described above for periods of at least 2 hr. Each block of stimuli resulting in 15 contractions took 5 min; between every two successive blocks there was a resting interval of 5 min. As shown in Fig. 2, which is representative of both experiments, the response was relatively constant for at least 2 hr. As a quantitative parameter of the response we took the mean height of the contractions of a block, omitting the first and the last contraction. The experiment (see Fig. 2) consisted of 14 of such blocks: the mean height was 2.3 ± 0.2 cm (s.d.). In another experiment with 7 blocks of contractions the mean was 4.8 ± 0.4 cm (s.d.). After two control blocks of contractions, a dose of

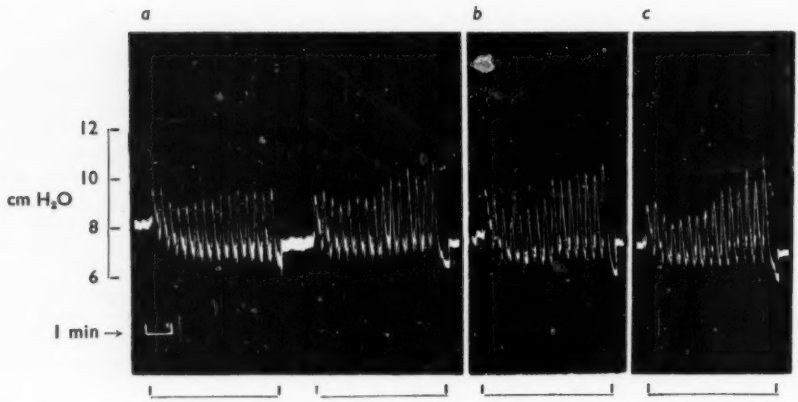

Fig. 2. Records of the vagally stimulated gastric contractions in the rat. For about 140 min the vagus nerve was stimulated repeatedly as follows: 5 min periods of stimulation (indicated by the horizontal brackets) were alternated with 5 min periods of rest, during which the kymograph was stopped. Each block of stimuli resulted in 15 contractions. *a*, The first and second block of contractions. The tone decreased only during the recording of the first block. *b*, Block of contractions after 1 hr.; *c*, after 2 hr.

the antagonist was injected in the course of the third block (Fig. 3), and stimulation was continued for 5 min after the injection. The inhibitory effect of the dose was taken as the height of the contractions obtained at the point of maximum inhibition: the height was then expressed as a percentage of the mean height of the control contractions. Because of the cumulative effect of the antagonist it was not possible to obtain more than one estimate in a single experiment. The second dose of atropine (4 μg) produced a more powerful inhibition than the first which was given 90 min earlier. At the time of the second dose the contractions had recovered their original height (Fig. 3).

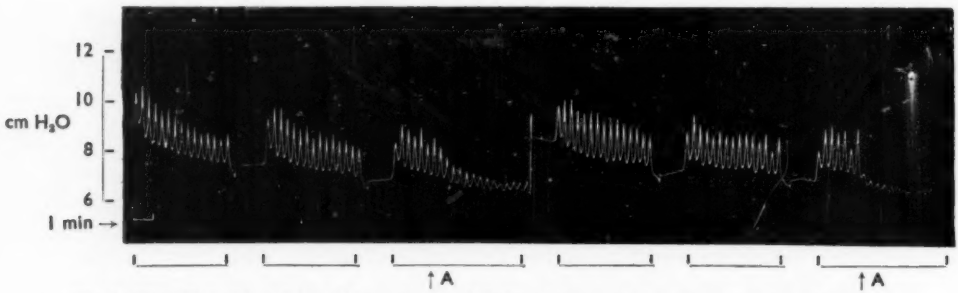

Fig. 3. The effect of two doses of 4 μg atropine (A) given intravenously with an interval of 90 min on the vagally stimulated gastric contractions. After two preceding control blocks of contractions the dose of atropine was injected in the course of the third. The second dose of atropine caused a stronger inhibition than the first made 90 min before, in spite of the fact that the preceding contractions had recovered their original height. The horizontal brackets indicate the periods of stimulation; the kymograph was stopped in the intervening 5 min rest periods.

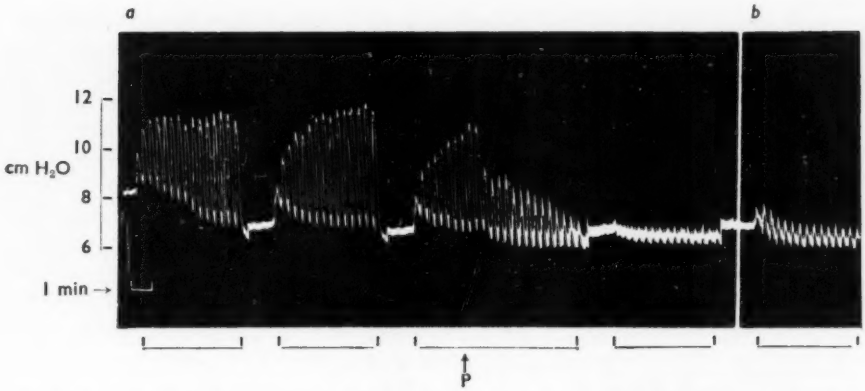

Fig. 4. Records of gastric contractions following vagal stimulation. *a*, The effect of 2 μg poldine (P) given intravenously. *b*, After a period of about 2 hr the inhibitory influence of poldine was still effective. The horizontal brackets indicate the periods of stimulation; the kymograph was stopped in the intervening 5 min rest periods.

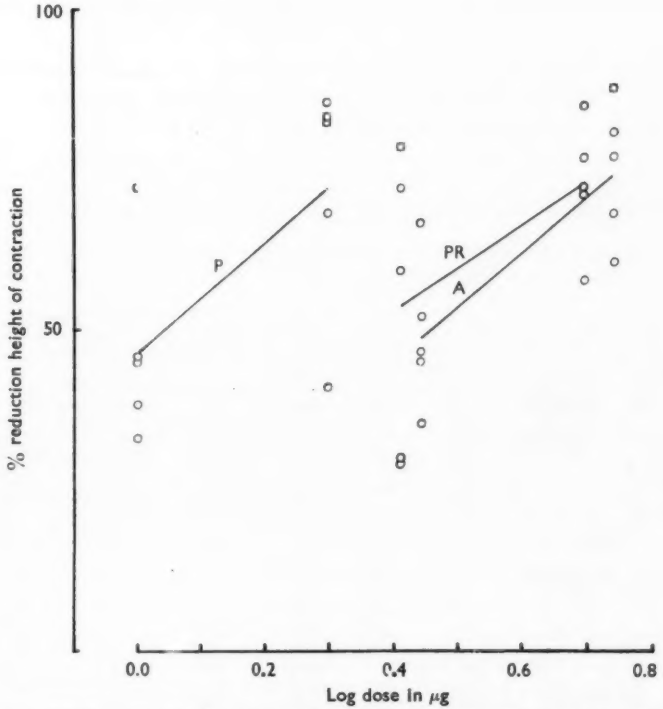

Fig. 5. The effects of atropine (A), propantheline (PR) and poldine (P) on the vagally stimulated gastric contractions in the rat.

The duration of effect of approximately equivalent doses of atropine, propantheline and poldine was found to increase in this order. Fig. 4 shows that after a period of about 2 hr a dose of 2 μg of poldine was still effective. Fig. 5 shows the log-dose-effect lines obtained for the three drugs investigated. The difference between the slopes of these lines was not significant at the 10% level. The slopes of the lines for atropine, propantheline and poldine were 82.48, 63.09 and 85.18, respectively. The activity-ratios of these drugs based on the doses giving a 50% reduction in height of contraction, as determined from the regression-lines, are given in Table 1.

TABLE 1

RATIOS FOR SPASMOLYTIC AND ANTI-ACETYLCHOLINE ACTIVITIES OF ATROPINE, PROPANTHELINE AND POLDINE

In brackets activities according to Acred *et al.* (1957)

Drugs	Spasmolytic activity on gastric contractions	Anti-acetylcholine activity on isolated guinea-pig ileum
Atropine	1·00	1·00
Propantheline	1·27	1·22 (2·09)
Poldine	2·60	1·00 (1·02)

Anti-acetylcholine activity. The anti-acetylcholine activity of propantheline and poldine was compared with that of atropine on the isolated guinea-pig ileum. The results are expressed as dose ratios and are given in Table 1.

TABLE 2

THE EFFECT OF ATROPINE ON THE HISTAMINE-STIMULATED ACID GASTRIC SECRETION

Roman numerals indicate the successive stimulations of acid secretion by a constant dose of histamine before and after atropine

Rat no.	Drug injected	Control estimation (lowest *p*H measured)			Estimations after atropine (difference between lowest *p*H measured and mean of control *p*H)		
		I	II	Mean	I	II	III
1	Atropine,	4·33	4·04	4·18	−0·06	+0·14	+0·08
2	11·2 μg	4·36	4·50	4·43	+0·05	+0·37	+0·43
3		4·88	4·73	4·80	−0·67	−0·12	−0·66
4		4·28	4·53	4·40	+0·36	+0·42	−0·22
5		3·73	3·77	3·75	−0·07	0·00	—
Mean					−0·07	+0·16	−0·09
6	Atropine,	4·22	3·78	4·00	−0·38	−0·13	+0·04
7	500 μg	3·82	4·12	3·97	−0·04	+0·23	+0·42
8		4·56	4·38	4·47	−0·22	−0·53	+0·35
9		5·03	4·63	4·83	−0·09	+0·14	+0·47
10		3·90	4·01	3·95	+0·29	+0·52	—
11		4·53	4·67	4·60	+0·64	+0·74	+0·90
12		4·16	4·45	4·30	+0·18	+0·40	+0·53
13		4·35	4·08	4·21	+0·02	+0·05	—
14		5·28	5·17	5·22	−0·26	−0·23	−0·07
Mean					+0·01	+0·16	+0·37

Acid gastric secretion. Starting after two control determinations (the response to the first dose of histamine in each case was neglected), the dose of the drug under test was injected intravenously about 3 min before the constant dose of histamine. Table 2 shows the results of experiments on the effect of atropine on acid secretion stimulated by successive, constant doses of histamine. Friedman's ranking test (Friedman, 1937) was applied to results obtained before and after administration of 11.2 μg of atropine (twice the dose which inhibited gastric contractions by 75%). This dose of atropine did not produce a significant ($P>0.05$) effect on acid secretion. Likewise, atropine in a dose of 500 μg was without effect on the acid secretory response to the first and second doses of histamine ; however, it did reduce ($P<0.05$) the effect of the third dose of histamine. The third dose was given at about 80 min after the administration of atropine.

Table 3 gives the results of experiments on the effect of propantheline and of poldine on histamine-stimulated acid secretion. In nearly all these experiments two

TABLE 3

THE EFFECT OF PROPANTHELINE AND POLDINE ON THE HISTAMINE-STIMULATED ACID GASTRIC SECRETION

Roman numerals indicate the successive stimulations of acid secretion by a constant dose of histamine

Rat no.	Control estimations (lowest *p*H measured) I	II	Mean	Drug injected	Estimations after injection (difference between lowest *p*H measured and mean of control *p*H) I	II	Drug injected	Estimations after injection (difference between lowest *p*H measured and mean of control *p*H) I
15	3·92	4·18	4·05	Propanthel-	+0·34	−0·05	Propanthel-	−0·27
16	4·02	3·63	3·82	ine 5·2 μg	−0·05	+0·68	ine 10·4 μg	+0·02
17	3·73	3·78	3·75		+0·05	+0·37		+0·38
18	3·55	3·53	3·54		+0·24	+0·38		+0·38
19	4·50	4·40	4·45		−0·35	+0·21		—
Mean					−0·04	+0·31		+0·12
20	4·04	4·12	4·08	Poldine	+0·08	−0·01	Poldine	+0·09
21	3·62	3·47	3·54	2 μg	+0·48	+0·13	10 μg	+0·20
22	4·03	4·03	4·03		+0·08	+0·20		+0·10
23	3·77	3·65	3·71		+0·21	+0·22		+0·07
24	3·73	—	—		+0·31	—		+0·34
Mean					+0·23	+0·13		+0·16

doses of the compound under test were injected. The first dose of each compound was that which reduced gastric contractions by approximately 75%. The dose of propantheline was then increased by twofold and, of poldine, by fivefold. These increased doses constituted the second dose of each compound.

Application of Friedman's ranking test shows that propantheline did not diminish the acid secretory response to the first dose of histamine which was injected immediately after the dose of propantheline. However, when the second dose of

histamine was injected after the propantheline had been allowed to act for 40 min, the acid secretory response was found to be significantly reduced ($P<0.05$). A second double dose of propantheline had no further effect on the response. Poldine in doses of 2 and 10 μg had no significant effect ($P>0.05$) on the histamine-stimulated acid secretion.

DISCUSSION

It is still an open question whether atropine and atropine-like drugs have a specific inhibitory effect or not on histamine-stimulated acid gastric secretion. Code (1951) points out that the size of the dose of atropine does not appear to be related quantitatively to the effect, that *in vivo* atropine never seems to abolish the effect of histamine, particularly in man. Polland (1930) found that the output of acid may be reduced, but the changes were small. Sometimes none were noted (Lim, Matheson & Schlapp, 1923). Atkinson & Ivy (1938) reported that toxic effects may be present before significant inhibition occurs.

Many investigators have found that in dogs atropine has an inhibitory effect on acid gastric secretion stimulated by histamine. However, the experiments of Janowitz & Hollander (1956) in dogs demonstrated clearly that the effect of 0.1 mg/kg atropine was delayed, since it appeared one hr after administration of the dose; thereafter the effect increased with time. Only a toxic dose of 0.4 mg/kg caused a reduction of the acid output in the first hr.

In the present experiments on rats 2.84 μg atropine was required for a 50% inhibition of the gastric contractions induced by vagal stimulation. Propantheline was 1.27 times, and poldine 2.6 times, more active than atropine. Poldine is about twice as active *in vivo* as *in vitro*, as judged by its anti-acetylcholine effect on isolated guinea-pig ileum. Poldine has the same anti-acetylcholine activity as atropine, which confirms the results of Acred, Atkins, Bainbridge, Brown, Quinton & Turner (1957). These authors reported that the anti-acetylcholine activity of propantheline as measured on the cat blood pressure, pupil diameter and salivary flow was much less than the anti-acetylcholine activity of propantheline on the isolated guinea-pig ileum. Nevertheless, they obtained a higher activity ratio for propantheline/atropine than that found in the present experiments.

A dose of atropine, twice as large as the dose which reduced gastric contractions by 75%, had no apparent effect on the histamine-stimulated acid gastric secretion. The same result was obtained with poldine in a dose which inhibited gastric contractions by approximately 75%. In a similar experiment with propantheline (also given in a dose which inhibited gastric contractions by approximately 75%) no inhibition of acid secretion was found after the first subsequent dose of histamine; however, the response to the second dose of histamine was probably diminished ($P<0.05$). Gastric contractions were diminished within a few seconds of injecting the drug, and therefore well within the period of 3 min elapsing between the injection of the drug and the administration of histamine.

A very high dose of atropine (500 μg) caused a delayed inhibitory effect, and only the stimulating effect of the third dose of histamine, injected about 80 min after the

atropine, was significantly reduced. Delayed inhibition was also seen after propantheline. It was possible that the delayed inhibition produced by atropine and propantheline was due not to the drug itself but to some metabolite. Injection of a double dose would then be expected to cause the formation of more of this hypothetical metabolite, leading to an increased inhibitory effect. The results of the experiments with propantheline shown in Table 3 did not support this hypothesis.

The fact that a second double dose of propantheline failed to increase the delayed inhibition suggested that the drug was not exerting a direct toxic effect.

Hence we concluded that the delayed inhibition was neither a specific anti-muscarine effect nor a manifestation of a direct toxic effect of atropine or propantheline.

Since the doses of atropine (or propantheline) and histamine were injected at regular intervals, it is possible that the non-specific suppressing effect on the acid secretion arose from the combined administration of these agents. It is known that both affect the blood vessels, and, moreover, Thompson & Vane (1953) have reported that a decrease of the gastric blood flow caused a decrease of the acid gastric secretion. Nordgren & Öbrink (1957) suggested that any substance that interferes in either direction with the blood flow in the gastric mucosa would alter the normal relationship between the volume output and acidity. They thought that the action of atropine on the acidity regulation is dependent on its effect on the blood flow through the gastric mucosa.

Experiments which permit the study of the combined effects of atropine and histamine on the gastric blood flow might therefore be helpful in elucidating this non-specific action on the acid secretion.

We are grateful to Professor S. E. de Jongh, University of Leiden, for his critical comments on this paper. We wish to thank Mrs H. Jansen-Otten and Mr R. Charles for carrying out a part of the experiments described.

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STUDIES ON THE SLOW CONTRACTION OF SMOOTH MUSCLE PRODUCED BY HUMAN PLASMA

BY

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The slow contraction of smooth muscle produced by human plasma becomes less potent after storage of the plasma for six months at room temperature and after processing of the plasma with kaolin. These observations fall into line with the fact that 40% of the initial smooth-muscle-contracting activity of the plasma can be accounted for in the deposit which is formed during the six-months period of storage, and also with the fact that the recovery of G acid from human plasma treated with kaolin is about 50% of the yield which is obtained from the same plasma before kaolin treatment.

Plasma is known to produce a quick contraction of the isolated guinea-pig ileum which is due to the presence of histamine or 5-hydroxytryptamine. It has recently been shown that after dialysis, which removes these agents, certain plasmas produce a delayed (at least 15 sec), slowly developing contraction of the ileum which is probably due to the formation of kallidin (or bradykinin) due to activation, by dilution, of an enzyme in plasma which releases it from a precursor which is also present (Schachter, 1956).

When larger volumes of human plasma are tested, evidence of another agent producing a delayed contraction appears. In this case there is a delay of only 4 to 10 sec after the addition of plasma to the isolated ileum, which then shortens fairly quickly, the response reaching a maximum within 30 sec. Recently, Gabr (1956) described the isolation from human plasma of a fatty acid which has a similar effect on the guinea-pig intestine. The present paper deals with the slow contraction produced by pooled plasma treated with kaolin (Maizels, 1944) and by pooled plasma stored at room temperature over a period of six months (Allen, Sykes, Enerson, Moulder, Elghammer, Grossman, McKeen & Galluzzi, 1950). The effect of pooled plasma on this contraction has also been studied.

In the method in which plasma is treated with kaolin, fibrinogen is removed from citrated plasma by adsorption, so that a fibrinogen-free plasma results which will not clot on filtration. Transfusion with this material has been particularly free from reactions, which suggests that kaolin may remove toxic substances.

According to Allen *et al.* (1950), pooled plasma stored in the liquid state without preservatives at room temperature for six months or longer before use produced no cases of homologous serum jaundice on transfusion when this was the only product administered. The virus of homologous serum jaundice in pooled plasma, which

has been stored in a liquid state at room temperature for six months, becomes attenuated and seldom if ever transmits the disease (Allen, 1952). Plasma stored in this manner for 18 to 24 months appears as effective for nutritive purposes as when stored for six months or less. These authors reported an over-all reaction to plasma stored at room temperature which was less than that to whole blood transfusion. The decreased reaction rate after the use of kaolin-treated plasma and room-temperature-stored plasma prompted the study of their effect on smooth muscle.

METHODS

Preparation of citrated plasma for storage at room temperature. Plasma was prepared from outdated blood (2 weeks or older). It was separated from the sedimented red cell mass and then stored at room temperature. Each blood transfusion was drawn in anticoagulant solution containing 2.2% trisodium citrate, 0.8% citric acid and 2.45% dextrose in pyrogen-free water (15 ml. solution for each 100 ml. blood). The blood was stored at 4° C to 6° C until transfused as such or converted to plasma, and was generally pooled with plasma from 10 donors. The plasma was then siphoned into standard M.R.C. plasma transfusion bottles under aseptic conditions. This plasma was stored at room temperature without preservatives. The plasma was prepared in a laboratory located in the same building as the donor centre. The temperature of the room in which the plasma was stored was recorded daily. Temperatures recorded during the period of storage varied from 14° C to 34° C. During room-temperature storage, monthly samples were taken for sterility control, chemical analysis and assay on the isolated guinea-pig ileum.

Isolation of G acid from the deposit formed in plasma stored at room temperature. Sterile human plasma slowly forms a precipitate as it ages, about 250 to 300 mg of precipitate per 100 ml. plasma being formed in six months. Chemically the deposit is similar in composition to the deposit which is formed in Seitz-filtered serum (Francis, Harrison & Picken, 1944). It consists of approximately the same amounts of calcium soaps, cholesterol and calcium phosphate. The percentage of nitrogen (13%) is, however, much higher than in the case of serum (1%), a fact which may be attributed to the clotting components which are readily denatured during storage at room temperature.

The precipitated material from twenty 500-ml. M.R.C. plasma bottles, each of an average age of 6 months, was collected by centrifugation and washed three times with distilled water, the precipitate being compacted by centrifugation after each washing. The compacted material was then dried to constant weight in a vacuum desiccator, acidified with concentrated hydrochloric acid and the liberated fatty acids were extracted with ether. After the removal of ether under reduced pressure, the ether extract was neutralized with 0.01 N sodium hydroxide. The material obtained was then processed according to the method of isolation of G acid from human plasma (Gabr, 1956), as follows:

Step 1. Removal of material insoluble in acetone.
Step 2. Chromatography on alumina (Savory & Moore).
Step 3. Acetic acid precipitation.
Step 4. Ether extraction and crystallization.

The crystalline product (4 mg, m.p. 34° C) has been chemically and biologically identified with G acid.

Processing of citrated plasma with kaolin. Colloidal kaolin (Leather Trades Specification) was placed in a 2-litre plasma Winchester (200 g/2 litre of pooled plasma), 10 ml. of saline was then added, and the whole sterilized by autoclaving at 20 lb./sq. in. for 1 hr. The plasma was then transferred from the Winchester bottle to the kaolin, left at 2° C for a day, shaken again and then left for a further 5 days to settle. The plasma was then siphoned into standard M.R.C. plasma transfusion bottles under aseptic conditions. Samples of pooled plasma were drawn from each Winchester before and after mixing with kaolin, for sterility

control, chemical analysis and assay on the isolated guinea-pig ileum. The plasma bottles were then left to stand at room temperature for 6 months in a place protected from direct sunlight as in the previous experiments. During storage at room temperature, monthly samples were taken for sterility control, chemical analysis and assay on the isolated guinea-pig ileum.

Sterility control. The neck and cap of the M.R.C. plasma bottle were flamed, and a 1 ml. sample was inoculated into 5 ml. of molten agar (45° C to 48° C). The agar bottles were then sloped on a glass rod and left to solidify at room temperature for 3 days, then incubated for 4 days at 37° C. The tests were read and infected bottles were discarded.

During room-temperature storage of the plasma, the following 2 tests were carried out at monthly intervals. Under sterile conditions, 10 ml. of plasma was drawn from the M.R.C. bottle and inoculated in 50 ml. of each of the following two media: (1) broth and (2) brewer's. In both tests incubation was carried out for 3 days at room temperature and 4 days at 37° C. The tests were read and the infected bottles were discarded.

Biological assay. One M.R.C. plasma bottle siphoned from each pool (normal plasma and kaolin-treated plasma pools) was transferred to ampoules, each holding 10 ml. of the plasma and dried from the frozen state by "Edwards Model 3PSA Centrifugal Freeze-Dryer" on the same day when the plasmas were distributed into the M.R.C. bottles. The moisture content of the product was then reduced to less than 1% by secondary desiccation over phosphorus pentoxide under high vacuum. The ampoules were then filled with dry nitrogen and sealed.

At monthly intervals a liquid sample drawn from each pool and its duplicate dried sample from the same pool before room-temperature storage were assayed on the isolated guinea-pig ileum. The determinations on the isolated guinea-pig ileum were made using a chamber of 55 ml. capacity filled with oxygenated Tyrode solution, the standard of reference being the reconstituted dried specimen taken from the same plasma pool, on the first day of storage. Plasma in doses of 2 to 4 ml. added to the Tyrode solution in the isolated organ bath caused contraction of the guinea-pig ileum after a latent period varying from 4 to 10 sec, depending on the sensitivity of the preparation. After the plasma had been in contact with the preparation for 30 sec, the preparation was washed by allowing the test fluid to flood over the rim of the organ bath. The contraction persisted after washing, slowly returning to the base-line. This took from 1 to 2 min. It is important to note that freeze-drying had no significant effect on the slow contraction of the isolated guinea-pig ileum, produced by citrated plasma (Fig. 1). The composition of Tyrode solution used as the test fluid in grams per litre is as

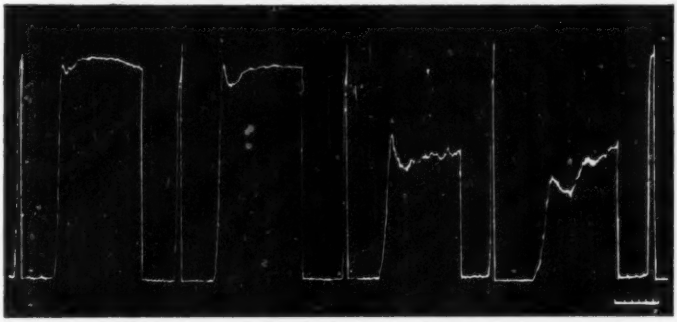

Fig. 1. The response of the isolated guinea-pig ileum to (1) 5 ml. liquid plasma, (2) 5 ml. reconstituted freeze-dried plasma, (3) 2 ml. reconstituted freeze-dried plasma, (4) 2 ml. liquid plasma, and to 0.2 μg acetylcholine (not numbered).

follows: 8.0 g sodium chloride, 0.2 g potassium chloride, 0.2 g calcium chloride, 0.01 g magnesium chloride, 0.05 g sodium dihydrogen phosphate, 1.0 g sodium bicarbonate and 1.0 g dextrose.

Chemical studies. Estimation of the plasma *p*H was made by the glass electrode technique, total plasma protein and non-protein nitrogen by micro-Kjeldahl.

RESULTS

Pooled plasma. Pooled plasma is generally less active on the isolated guinea-pig ileum than single donor plasma. This effect seems to depend on the size of the plasma pool; plasma pooled from a smaller number of samples was generally more active than that derived from a larger number. Thus, plasma pools derived

Fig. 2. The response of the isolated guinea-pig ileum to (1) 2 ml. pooled plasma, (2) 2 ml. plasma (blood bottle no. 896, blood group B), (3) 2 ml. plasma (bottle no. 61, blood group AB), (4) 2 ml. plasma (bottle no. 2342, blood group A), (5) 2 ml. plasma (bottle no. 2354, blood group A), (6) 2 ml. plasma (bottle no. 2357, blood group A), (7) 2 ml. plasma (bottle no. 2359, blood group A), (8) 2 ml. plasma (bottle no. 6102, blood group O), (9) 2 ml. pooled plasma, and to 0.2 μg acetylcholine (not numbered).

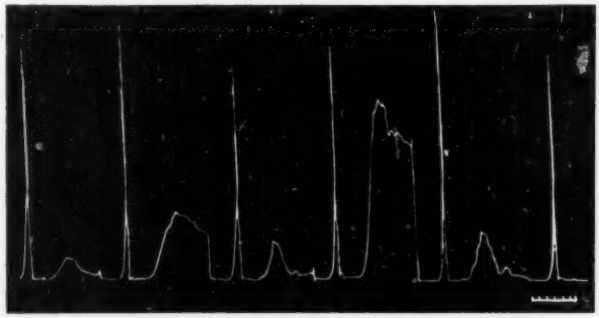

Fig. 3. The effect of the size of plasma pool on the slow contraction of the isolated guinea-pig ileum produced by pooled plasma. Response of the guinea-pig ileum to (1) 4 ml. pooled plasma (plasma pool derived from 10 blood bottles), (2) 4 ml. pooled plasma (plasma pool derived from 7 blood bottles), (3) 4 ml. pooled plasma (plasma pool derived from 10 blood bottles), (4) 4 ml. pooled plasma (plasma pool derived from 3 blood bottles), (5) 4 ml. pooled plasma (plasma pool derived from 10 blood bottles), and to 0.2 μg acetylcholine (not numbered).

from 10 donations of blood were found to be less active on the isolated ileum than plasma pools derived from 7 donations of blood. Again, the latter were less active than those derived from 3 blood donations. All of these plasmas were prepared from different group donors but always including a group B blood in each pooled sample. When the samples were derived from 10 or more donations of blood, the onset of muscle contraction was much slower, and the maximum contraction often declined within a few seconds. Fig. 2 shows the effect of pooling on the slow contraction of smooth muscle produced by plasma from a single donor. No relation could be found between the toxicity of pooled plasma for smooth muscle and the group types of the blood bottles from which the plasmas were separated. Fig. 3 shows the effect of the size of pool on the slow contraction of the isolated guinea-pig ileum produced by pooled plasma. It may be interesting to note that the activity of pooled plasma on smooth muscle decreased slightly after standing for one week at room temperature prior to freeze-drying.

Plasma stored at room temperature. The smooth-muscle-stimulating activity, as measured on the guinea-pig ileum, of liquid pooled plasma stored at room temperature for 6 months decreases. The decrease is gradual and varies from one batch

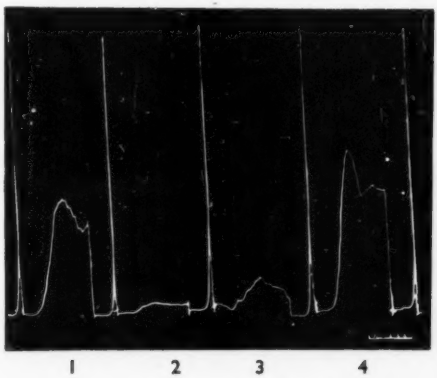

Fig. 4. The effect of room-temperature storage of plasma on the slow contraction of the isolated guinea-pig ileum produced by normal plasma and kaolin-treated plasma. Responses to (1) 3 ml. reconstituted dried kaolin-treated plasma (pool no. 241), (2) 3 ml. kaolin-treated liquid plasma stored at room temperature for six months (pool no. 241), (3) 3 ml. liquid plasma stored at room temperature for six months (pool no. 230), (4) 3 ml. reconstituted dried plasma (pool no. 230), and to 0.2 μg acetylcholine (not numbered).

to another. The contraction starts more slowly and relaxation takes much longer in spite of repeated washings of the organ bath with Tyrode solution. The plasma becomes more turbid and does not clot after the addition of calcium chloride. Fig. 4 shows the effect of storage on the slow contraction produced by normal liquid plasma on the isolated guinea-pig ileum.

Average results of 5 pools show that the *p*H of normal liquid plasma decreases from a normal value of 7.05 to 6.80 over the six-months period. The total protein

decreases from a normal value of 5.8% to 5%. This decrease in total protein is more marked in this series of experiments than in those experiments reported by Taylor, Lozner, Davidson, Tagnon & Newhouser (1944), in which glucose and merthiolate were added to liquid plasma for preservation. The non-protein nitrogen of liquid plasma does not show significant alteration during storage at room temperature (Table 1). Cultures for bacterial growth were uniformly negative.

TABLE 1

AVERAGE VALUES OF MEASUREMENTS MADE ON NORMAL LIQUID PLASMA STORED AT ROOM TEMPERATURE OVER A PERIOD OF SIX MONTHS (5 POOLS)

0 = Baseline values which were also given by the freeze-dried duplicate specimens after reconstitution with distilled water

Period of storage (months)	*p*H ± s.d	Total protein (g/100 ml.) ± s.d.	Non-protein nitrogen (mg/100 ml.) ± s.d.
0	7·05 ± 0·17	5·8 ± 0·17	21·7 ± 1·4
1	7·05 ± 0·32	5·55 ± 0·17	22·7 ± 1·3
2	7·00 ± 0·1	5·05 ± 0·45	21·4 ± 3·9
3	6·95 ± 0·1	4·8 ± 0·15	21·7 ± 0·4
4	7·00 ± 0·24	4·9 ± 0·1	23·4 ± 2·0
5	6·9 ± 0·1	5·1 ± 0·25	22·4 ± 2·0
6	6·8 ± 0·16	5·0 ± 0·3	21·9 ± 2·4

Analysis of the deposit which is formed in liquid plasma during the six-months period of storage at room temperature showed that about 10% of the original smooth-muscle-stimulating activity of the plasma can be recovered from the deposit. Since the total recovery of this activity is about 25% of that of the original active material (Gabr, 1956), one may conclude that the deposit which is formed during storage at room temperature retains 40% of the original smooth-muscle-stimulating activity of the plasma. This conclusion may explain the marked decrease in the effect of liquid plasma on the isolated guinea-pig ileum after the six-months period of storage at room temperature (Gabr & Aly, 1959).

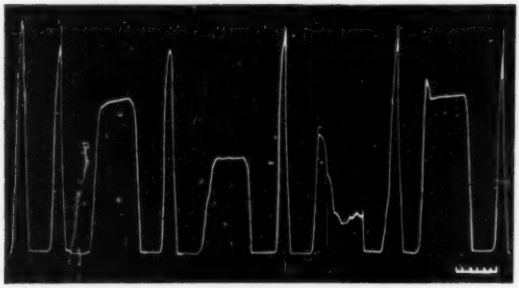

Fig. 5. The response of the isolated guinea-pig ileum to liquid plasma before and after processing with kaolin. Responses to (1) 4 ml. reconstituted freeze-dried plasma, (2) 4 ml. reconstituted freeze-dried plasma which had been treated with kaolin, (3) 4 ml. kaolin-treated plasma, kept at room temperature for one week, in the liquid state, (4) 4 ml. reconstituted freeze-dried plasma, and to 0.2 μg acetylcholine (not numbered). All specimens were taken from the same plasma pool.

Kaolin-treated plasma. Processing of pooled plasma with kaolin decreased its effect on the isolated guinea-pig ileum. The effect of kaolin was more marked at room temperature than at 2° C. Like normal plasma, kaolin-treated plasma became less active on the isolated guinea-pig ileum after storage for six months at room temperature. The final product was less active than normal plasma stored under the same conditions without preservatives. Kaolin-treated plasma is clearer than normal plasma, and develops no turbidity during storage in spite of the relatively big variation in room temperature.

Fig. 5 shows the response of the isolated guinea-pig ileum to liquid plasma before and after processing with kaolin. The effect of storage at room temperature on the slow contraction produced by kaolin-treated plasma is shown in Fig. 4.

Average results of 5 pools showed that processing with kaolin does not significantly affect the *p*H or the non-protein nitrogen of the plasma. There was, however, a marked drop in total protein (from a normal value of 6.2% to 4.9%). The *p*H of kaolin-treated plasma increased slightly from a normal value of 7.0 to 7.2 over the six-months period of storage at room temperature. The total protein decreased from an average value of 4.9% to 4.5%. The non-protein nitrogen of kaolin-treated plasma did not show significant alteration during the same period of storage (Table 2). Cultures for bacterial growth were uniformly negative.

TABLE 2

AVERAGE VALUES OF MEASUREMENTS MADE ON KAOLIN-TREATED LIQUID PLASMA STORED AT ROOM TEMPERATURE OVER A PERIOD OF SIX MONTHS (5 POOLS)

0 = Baseline values which were also given by the freeze-dried duplicate specimens after reconstitution with distilled water

Period of storage (months)	*p*H ± s.d.	Total protein (g/100 ml.) ± s.d.	Non-protein nitrogen (mg/100 ml.) ± s.d.
Before treatment	6·9 ±0·2	6·2±0·24	22·7±3·1
0	7·0 ±0·18	4·9±0·23	21·7±0·9
1	7·1 ±0·14	4·9±0·17	21·0±0·7
2	7·2 ±0·1	4·9±0·17	22·9±1·1
3	7·25±0·12	4·9±0·25	22·9±1·2
4	7·3 ±0·12	4·7±0·15	22·9±1·0
5	7·3 ±0·24	4·6±0·25	22·9±1·1
6	7·2 ±0·12	4·5±0·19	22·9±1·1

The deposit which is formed in kaolin-treated plasma during the six months of storage at room temperature is much similar in its chemical composition to the deposit which is formed in human serum under the same conditions of storage. The recovery of smooth-muscle-stimulating activity from this deposit by the method described is very small when compared to that of untreated plasma.

DISCUSSION

Some of the reactions which may occur following the administration of human plasma are characterized by violent pain in the back, vomiting and defaecation. These reactions have been attributed to certain pharmacologically active substances, the presence of which in serum and plasma has been demonstrated by Reid &

Bick (1942a & b). Lozner & Newhouser (1944) reported a decreased reaction rate upon prolonged storage of normal citrated plasma at room temperature. Clinical experience with the material stored at room temperature showed that younger plasma was followed by reactions more often than older plasma, a phenomenon which was attributed by the authors to the disappearance of some mildly toxic labile constituent. There is also some evidence that processing of the plasma with kaolin (Maizels, 1944) removes some of the pharmacologically active substances from the plasma, and this was borne out clinically by the fact that kaolin plasma seemed remarkably free from all reactions. Although a number of pharmacologically active substances have been isolated from or identified in plasma, the relationships between these pharmacologically active substances to the reactions described have never been proved clinically. The results cited in this paper show that storage at room temperature decreases the slow contraction of smooth muscle produced by citrated plasma. Processing of citrated plasma with kaolin produces a similar effect. This effect may arise from partial precipitation of the causal factors during storage at room temperature of the plasma in the first case and partial adsorption of the causal factors on kaolin in the second case. These conclusions are borne out biologically by the fact that 40% of the starting smooth-muscle-contracting activity of citrated plasma could be accounted for in the deposit which is formed during the six-months period of storage at room temperature in the first case, and by the fact that the recovery of G acid from kaolin plasma is about 50% of the yield obtained from the same plasma before kaolin treatment in the second case. Attempts to recover G acid from the kaolin used in this method were unsuccessful.

The decrease in the total protein concentration of the plasma during storage at room temperature cannot account for the lower activity of the final product on the isolated guinea-pig ileum, since it was found that the response of this preparation to different plasmas was not parallel to their total protein content. The changes in *p*H and non-protein nitrogen of the plasma during storage at room temperature and after kaolin treatment were too slight to influence the response of the isolated guinea-pig ileum to the slow contracting principle of the plasma.

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EFFECTS OF DRUGS ON PARTIALLY AND COMPLETELY DENERVATED SKELETAL MUSCLE OF THE FROG

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The sensitivity of completely denervated and the innervated end-plate regions of partially denervated frog's sartorius muscles to stimulation by drugs has been investigated. The drugs were tested on muscles isolated 12 to 39 days following denervation. On the basis of the results obtained most of the drugs could be put into one of two main groups. The first group included choline, tetramethylammonium, carbachol and decamethonium ; these drugs readily stimulated non-denervated muscles, and chronic denervation, either partial or complete, greatly increased the sensitivity of the muscles. The second group included pilocarpine and carbamate of (2-hydroxypropyl) trimethyl ammonium chloride (Bethanechol, U.S.P.). The muscles were relatively insensitive to stimulation by these two drugs. They could stimulate the muscles if applied in high enough concentrations, but the responses thus obtained could not be distinguished from responses produced by similar concentrations of sucrose and chronic denervation did not increase the sensitivity of the muscles to stimulation by these two drugs. Methacholine, like the drugs in the second group, was unable to stimulate the muscles in low concentrations, but chronic denervation rendered the muscles sensitive to stimulation by this drug. Tetraethylammonium presented a completely different pattern of activity which was consistent with a hypothesis that in low doses this drug stimulates skeletal muscle by causing a release of acetylcholine from nerve endings in the muscle.

It has been known for a long time that a denervated structure develops an increased sensitivity to many drugs in addition to its normal transmitter (Cannon & Rosenblueth, 1949). Recently it was reported (Frank, 1959) that the normally innervated end-plate regions of frog sartorius muscle fibres develop an increased sensitivity to their normal chemical mediator following denervation of another end-plate region on the same cells. In a subsequent report, Miledi (1960a) reached the opposite conclusion, although he did not adequately test for an increased sensitivity of innervated end-plates in partially denervated muscles. Because this point is relevant to any theory which attempts to explain the mechanism of denervation sensitization, it was decided that further work was warranted. In the present study on the sensitivity of partially and completely denervated frog sartorius muscles, several nicotinic and muscarinic drugs were tested.

METHODS

The methods used were described previously in detail (Frank, 1959). In the frog sartorius muscle the individual fibres extend the full length. There are two end-plate regions, one

proximal and one distal, with a distinct nerve supply to each, and most fibres have at least one neuromuscular junction in each end-plate region (Katz & Kuffler, 1941).

One sartorius muscle of each frog was denervated several days prior to testing; the sartorius muscle of the other leg served as a control. For complete denervation the whole nerve was severed at its site of entry into the muscle. For partial denervation, all the nerve branches in the muscle running towards the distal end were cut. Nerve section was done under a dissecting microscope. Only results obtained with muscles denervated 12 to 39 days prior to testing were used to determine the effects of denervation.

For testing, both sartorii were removed from the frog, leaving proximal ends attached to the pelvic girdle. The muscle pair thus formed was mounted vertically in a bath with the distal end of each muscle attached by a nylon thread to a critically balanced isotonic lever above the bath. The bath was drained or filled from below to permit the test solution to cover the proximal end-plate region without touching the distal end-plate zone. For testing, all drugs were dissolved in a solution containing the following salts (in mM): sodium chloride, 111.3; potassium chloride, 2.5; calcium chloride, 1.08; sodium bicarbonate, 2.4; sodium dihydrogen phosphate, 0.087; glucose, 11.1. The drug concentrations in the test solutions were increased in steps of approximately threefold, and, for ease in comparing sensitivity to various drugs, concentrations are reported in mM. The proximal end-plate regions of both muscles were exposed simultaneously to a test solution for at least 30 sec, and between tests the muscles were kept in a drug-free solution for at least 15 min. No more than two drugs were tested on any one muscle pair. No attention was paid to the order of drug application, with the exception of decamethonium, which was always the last drug tested on any muscle pair.

The tests were designed to find the minimum drug concentration which would produce a discernible response in the muscle. This was considered the threshold concentration, provided that the effect was reproducible and that the muscle responded to the next higher drug concentration.

In each test the thresholds for the two sartorius muscles from a single frog were determined with the muscles mounted in a single bath. Since only one muscle from each frog was chronically denervated, the non-denervated sartorius muscle from the other leg served as a control. Using the results thus obtained, a separate threshold ratio was determined for each muscle pair, and these are listed in the Tables under the columns headed "Ratio (C/D)."

After every experiment with a partially denervated muscle it was examined under a dissecting microscope to confirm that the nerve section had been properly performed, to see if the nerve supply to the distal end-plate region had regenerated, and to ascertain that the nerve supply to the proximal end-plate region was viable. The results obtained with muscles showing signs of nerve regeneration are reported separately.

RESULTS

The sensitivity of sartorius muscles from different frogs to the same drug varies greatly. Thus it was previously reported (Frank, 1959) that the acetylcholine thresholds of sartorius muscles from different frogs may differ by a factor of 1,000 times. This factor was smaller for each drug reported here, although differences were still present and made it necessary to establish some criterion for denervation sensitization. It is generally assumed that the homologous muscles from the two sides of an animal are equally sensitive to applied drugs, and thus if only one is chronically denervated the other can be used as a control. The thresholds of acetylcholine for 24 pairs of sartorius muscles in which the innervation remained intact were previously determined (Frank, 1959), and the threshold sensitivities of pairs of innervated muscles reported here also were tested for all but one of the drugs (see below). In all tests the homologous muscles from the same frog had the same threshold.

Another indication that homologous muscles were equally sensitive to applied drugs was the effect of regeneration on the sensitivity of partially denervated muscle. When the nerve supply to the distal end-plate region had regenerated, the difference in the drug sensitivity of the proximal end-plate regions in the denervated and control muscles tended to disappear. This always occurred when testing thresholds of acetylcholine (Frank, 1959) and usually occurred when testing the thresholds of the other drugs included in the present study.

All soluble chemicals tested could produce a muscle response, provided that a large enough concentration was used. Since the drugs were added to the test solution for testing and since often extremely large drug concentrations were necessary, it was thought that the responses might have been produced by the tonicity of the

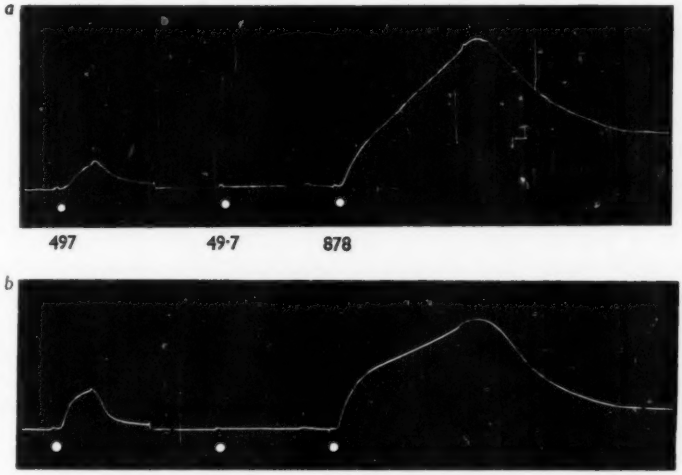

Fig. 1. Responses of frog sartorius muscles produced by the application of hyperosmotic sucrose-containing solutions to the lower halves of the muscles. (*a*) Partially denervated muscle; (*b*) control muscle from other leg of frog. Dots below records indicate point at which test solutions were added to the bath. Numbers between the records indicate the concentrations of sucrose (in mM). Small rectangular artifacts on the records were caused by emptying the bath.

test solutions rather than by the action of the drug. To test this possibility the response of the muscles to high concentrations of sucrose was tested. Responses produced by sucrose are shown in Fig. 1. Three pairs of muscles were thus tested, and, in all three, sucrose concentrations of 497 mM or larger produced a response. In two of these pairs a sucrose concentration of 49.7 mM failed to produce a muscle response.

In the experiments using drugs, any muscle which did not respond when tested with a concentration of 50 mM or more was considered to be relatively insensitive to the drug. All muscles responded to any drug tested in a concentration of 410 mM or greater, but for these the response would have to be attributed to the tonicity

of the test solution. Because of the responses to high osmotic concentrations, it was impossible to determine if a muscle was completely insensitive to the effect of a drug.

Choline chloride. It has long been known that choline can stimulate skeletal muscle, differing from acetylcholine only in being less potent. Eleven pairs of muscles were tested with choline (Table 1). These included 12 innervated control muscles. The most sensitive of these control muscles had a choline chloride

TABLE 1

CHOLINE CHLORIDE CONCENTRATIONS REQUIRED TO PRODUCE THRESHOLD CONTRACTIONS OF FROG SARTORIUS MUSCLES

Ranges given except where not applicable. n, number of muscle pairs; C/D, ratio of threshold doses for control and denervated muscle pairs except in the pairs without a chronically denervated muscle, when the ratio represents right sartorius (R)/left sartorius (L); >=greater than, indicates that the muscle did not contract, but higher drug concentrations were not tested

Preparation	n	Control (C) (mM)	Denervated (D) (mM)	Ratio (C/D)
No denervation	1	R, 7·16; L, 7·16		1
Complete denervation	5	7·16–>71·6	0·716–7·16	3–>100
Partial denervation	3	7·16–>71·6	2·15–7·16	3–>10
Partial denervation with regeneration	2	7·16	7·16	1

threshold of 7.16 mM. Using acetylcholine (Frank, 1959), the most sensitive muscles responded to a solution containing 5.37×10^{-4} mM, or a difference of about 10,000 times.

As would be expected, chronic complete denervation increased the sensitivity of frog sartorius muscles to stimulation by choline. Similarly the innervated end-plate regions of the partially denervated muscles also developed an increased sensitivity to choline stimulation. In the two examples in which the partially denervated muscles were observed to have a viable nerve supply to their distal end-plate regions following drug testing (Table 1), the partially denervated muscles and their controls did not differ in their sensitivity to choline.

Pilocarpine hydrochloride. This alkaloid has marked parasympathomimetic effects, but apparently is completely devoid of nicotinic properties. Five pairs of muscles were tested with solutions containing pilocarpine. Three of the muscles had been completely denervated 9, 13 and 34 days prior to testing. Two of these pairs were tested with a maximum pilocarpine concentration of 41 mM and did not contract. The other three responded when tested with a solution containing 410 mM; one of the pairs tested with 123 mM did not respond. There was no observed effect of complete denervation on the threshold or response to pilocarpine. In these tests pilocarpine had an effect and potency comparable to that of sucrose.

Tetramethylammonium chloride. In agreement with the known nicotinic properties of tetramethylammonium, Dale & Gasser (1926) demonstrated that denervated mammalian skeletal muscle developed an increased sensitivity to stimulation by this drug. Similar results were obtained in the present experiments. It was observed that both completely denervated frog sartorius muscles and the

TABLE 2

TETRAMETHYLAMMONIUM CHLORIDE CONCENTRATIONS REQUIRED TO PRODUCE THRESHOLD CONTRACTIONS OF FROG SARTORIUS MUSCLES

Ranges given except where not applicable. n, number of muscle pairs; C/D, ratio of threshold doses for control and denervated muscle pairs except in the pairs without a chronically denervated muscle, when the ratio represents right sartorius (R)/left sartorius (L); >=greater than, indicates that the muscle did not contract, but higher drug concentrations were not tested

Preparation	n	Control (C) (mM)	Denervated (D) (mM)	Ratio (C/D)
No denervation	1	R, 0·274; L, 0·274		1
Complete denervation	6	0·0913–0·913	0·0274–0·0913	3·3–10
Partial denervation	3	0·274–0·913	0·0913	3–10
Partial denervation	2	0·274	0·274	1
with regeneration	1	0·274	0·0913	3

innervated end-plate regions of partially denervated muscles had a lower threshold to stimulation by tetramethylammonium chloride than did control muscles (Table 2).

An interesting observation was that the proximal end-plate region of one of the partially denervated muscles had an increased sensitivity to tetramethylammonium stimulation, despite the fact that the nerve supply to the distal end-plate region had regenerated. This persistent increase in sensitivity in the presence of regeneration was never found in experiments with acetylcholine (Frank, 1959), but was observed with some of the other drugs used in the present study (see below).

Tetraethylammonium bromide. Although solutions containing tetraethylammonium readily stimulate frog sartorius muscles, both the type of contraction obtained and the effects of denervation on the response to this drug indicate that, at least in lower concentrations, it produces its stimulation by a mechanism of action different from that of the other drugs included in this study.

In the innervated muscle the typical threshold response produced by all the other active drugs studied was a slow, smooth shortening. In contrast, tetraethylammonium either produced no response or a violent twitching of the muscle (Fig. 2*b*).

Seven pairs of muscles, including a completely denervated muscle, were tested with tetraethylammonium (Table 3). In all but one of these, the control muscle was more sensitive than the denervated muscle to stimulation by this drug. Another result of the complete denervation was elimination of the violent twitching, the

TABLE 3

TETRAETHYLAMMONIUM BROMIDE CONCENTRATIONS REQUIRED TO PRODUCE THRESHOLD CONTRACTIONS OF FROG SARTORIUS MUSCLES

Ranges given except where not applicable. n, number of muscle pairs; C/D, ratio of threshold doses for control and denervated muscle pairs except in the pairs without a chronically denervated muscle, when the ratio represents right sartorius (R)/left sartorius (L); >=greater than, indicates that the muscle did not contract, but higher drug concentrations were not tested

Preparation	n	Control (C) (mM)	Denervated (D) (mM)	Ratio (C/D)
No denervation	1	R, 4·76; L, 4·76		1
Complete denervation	6	4·76–47·6	47·6–>47·6	<0·1–<1
	1	14·3	4·76	3
Partial denervation	3	14·3	0·476–4·76	3–30
	2	4·76, 47·6	4·76, 47·6	1
Partial denervation with regeneration	1	4·76	4·76	1

response of the denervated muscle to this drug being similar in appearance to the responses to all other stimulant drugs studied (Fig. 2*a*). The records obtained in the one case in which the completely denervated muscle had a lower threshold than its control are shown in Fig. 2.

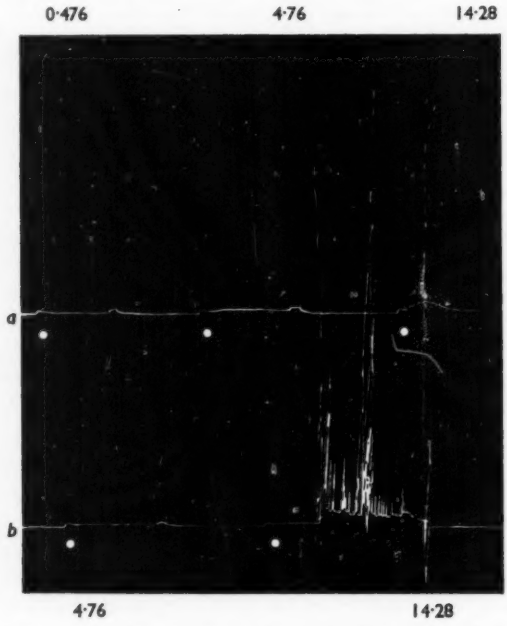

Fig. 2. Responses of frog sartorius muscles to the application of tetraethylammonium-bromide-containing solutions. (*a*) Completely denervated muscle; (*b*) control muscle from other leg of frog. 18 days post-denervation. Levers not aligned. Dots below records indicate point at which test solutions were added to the bath. Numbers above and below record indicate the tetraethylammonium concentrations (in mM). Small rectangular artifacts on the records were caused by emptying the bath.

The type of response produced by the application of tetraethylammonium to the innervated end-plate region of the partially denervated sartorius muscle was the same as produced in the control muscles. In contrast to the changes produced by complete denervation, 3 of the 5 partially denervated muscles had a lower threshold to stimulation by this drug than did their controls.

These results can best be explained if it is assumed that the stimulation produced by tetraethylammonium is brought about by the release of acetylcholine from the nerve endings in the muscle and only in the completely denervated muscle is a direct stimulation of the muscle fibres by this drug uncovered.

Bethanechol chloride, methacholine chloride, and carbachol chloride. These three closely related drugs presented an interesting spectrum of activities, with carbachol having marked nicotinic properties, bethanechol devoid of nicotinic activity and methacholine intermediate (Table 4).

TABLE 4

BETHANECHOL, METHACHOLINE, AND CARBACHOL CONCENTRATIONS REQUIRED TO PRODUCE THRESHOLD CONTRACTIONS OF FROG SARTORIUS MUSCLES

Ranges given except where not applicable. n, number of muscle pairs; C/D, ratio of threshold doses for control and denervated muscle pairs except in the pairs without a chronically denervated muscle, when the ratio represents right sartorius (R)/left sartorius (L); >=greater than, indicates that the muscle did not contract, but higher drug concentrations were not tested

Drug and preparation	n	Control (C) (mM)	Denervated (D) (mM)	Ratio (C/D)
Bethanechol				
Complete denervation	5	>5·09–>50·9	>5·09–>50·9	—
Partial denervation	1	>50·9	>50·9	—
Partial denervation with regeneration	1	>50·9	>50·9	—
Methacholine				
No denervation	2	R>51·2, 512 L>51·2, 512		—, 1
Complete denervation	6	>51·2	0·512–5·12	>10–>100
Partial denervation	3	>51·2	5·12–51·2	>1–>10
Partial denervation with regeneration	1	>51·2	>51·2	—
	2	>51·2	5·12	>10
Carbachol				
No denervation	3	R, 0·0548–0·164 L, 0·0548–0·164		1
Complete denervation	5	0·0548–0·548	0·00164–0·0164	10–100
Partial denervation	4	0·0164–0·548	0·00164–0·00548	10–100
Partial denervation with regeneration	1	0·0548	0·0548	1
	1	0·0548	0·0164	3·3

In none of the muscles of the seven pairs tested with bethanechol was a contraction obtained. Although 50.9 mM was the largest concentration tested, the effects of higher concentrations could have been attributed to osmotic effects.

In contrast, carbachol had marked nicotinic stimulant properties. It readily stimulated both control and denervated muscles, and in this it was the most potent drug included in the present study. Both the completely denervated muscles and the innervated end-plate regions of the partially denervated muscles had an increased sensitivity to stimulation by this drug.

An intermediate response pattern was obtained in tests with methacholine. All the control muscles were relatively insensitive to stimulation, but the chronically denervated muscles, both complete and partial, were sensitive to stimulation by methacholine. Even 2 of the partially denervated muscles in which the nerve supply to the distal end-plate region had regenerated had a threshold of 5.12 mM.

Decamethonium bromide. Zaimis (1951) showed that the response of denervated mammalian skeletal muscle to decamethonium was identical to its response to acetylcholine. In the present study only the stimulation produced by this neuromuscular blocking agent in low doses has been investigated. As can be seen from the results presented in Table 5, both completely denervated muscles and the innervated end-plate regions of the partially denervated muscles were sensitized to the stimulant action of this drug. Although at the start of these tests the possibility was considered that the muscles might become blocked by the decamethonium without showing any measurable signs of stimulation, this did not prove to be a

TABLE 5

DECAMETHONIUM BROMIDE CONCENTRATIONS REQUIRED TO PRODUCE THRESHOLD CONTRACTIONS OF FROG SARTORIUS MUSCLES

Ranges given except where not applicable. n, number of muscle pairs; C/D, ratio of threshold doses for control and denervated muscle pairs except in the pairs without a chronically denervated muscle, when the ratio represents right sartorius (R)/left sartorius (L); >=greater than, indicates that the muscle did not contract, but higher drug concentrations were not tested

Preparation	n	Control (C) (mM)	Denervated (D) (mM)	Ratio (C/D)
No denervation	1	R, 1·20; L, 1·20		1
Complete denervation	3	0·0718–0·239	0·00718	10–33
Partial denervation	3	0·239	0·00239–0·0239	10–100
	1	>23·9	0·239	>100
Partial denervation with regeneration	2	0·239	0·239	1

problem. However, in one pair of muscles the threshold for the control muscle was greater than 23.9 mM (Table 5), and, since this is considerably above all the other thresholds recorded, it is possible that in this one instance the muscle was blocked without a response being recorded.

DISCUSSION

Dale & Gasser (1926) divided drugs which act like acetylcholine into two groups, those which stimulated and those which failed to stimulate denervated mammalian muscles. They pointed out that the active drugs were characterized by also having nicotine-like effects on ganglion cells, and that chronic denervation had made the mammalian skeletal muscle comparable to muscles from amphibia and birds which are normally sensitive to stimulation by nicotinic drugs.

With two notable exceptions, the drugs tested in the present study fit the pattern that they described. Thus the chronically denervated muscles developed an increased sensitivity to stimulation by choline, tetramethylammonium, carbachol and decamethonium, all of which were relatively potent in stimulating the control muscles. On the other hand, pilocarpine and bethanechol were unable to stimulate either control or denervated muscles in reasonably low doses, and any stimulation obtained could be attributed to an osmotic effect comparable to that produced by sucrose solutions.

Methacholine presented an interesting exception to this pattern in that the control muscles were relatively insensitive to stimulation by this drug, whereas the denervated muscles had developed a marked sensitivity (Table 4).

The results obtained with tetraethylammonium differed from those obtained using the other drugs in almost every respect. Tetraethylammonium markedly antagonizes the blocking action of curare on neuromuscular transmission (Riker, 1953). Stovner (1958) concluded that tetraethylammonium produced its anticurare effect by increasing the amount of acetylcholine released by each nerve impulse. The present results also support the concept that tetraethylammonium can act by facilitating the release of acetylcholine from nerve endings in muscle. Thus the threshold response to tetraethylammonium is a violent twitching of the muscle (Fig. 2), as would be expected if there was a sudden release of acetylcholine from the nerve endings once the concentration of tetraethylammonium in the extracellular fluid reached a critical

level. In contrast, drugs such as acetylcholine or decamethonium which depolarize the end-plates by a direct action produce a smooth muscle shortening at threshold concentrations. This would be expected from the conditions of the present experiment which result in a gradual rise in the drug concentration in the extracellular spaces of the muscle. It is of interest that the stimulation by tetraethylammonium of the completely denervated muscle, in which the nerve endings have degenerated, results in a response similar in shape to that produced by drugs known to depolarize directly the muscle end-plates.

The hypothesis that tetraethylammonium can release acetylcholine from the nerve endings in muscle, and that this release can be brought about by lower doses than those needed to produce a measurable direct response, also explains the observed increase in threshold in most of the completely denervated muscles. However, in one instance the threshold for the completely denervated muscle was lower than that of its control (Fig. 2). This indicates that tetraethylammonium can directly stimulate the muscle and that the chronically denervated muscle can develop an increased sensitivity to this direct effect. The above hypothesis also would explain the observed increase in sensitivity of the innervated end-plate regions of some of the partially denervated muscles to tetraethylammonium, since it has previously been shown that these regions develop an increased sensitivity to direct depolarization by acetylcholine (Frank, 1959). Since the response of these innervated regions of partially denervated muscles would not depend only on their acetylcholine thresholds but also on the amounts of acetylcholine released by the tetraethylammonium from the nerve endings, it is not surprising that two of the five partially denervated muscles did not show a lower tetraethylammonium threshold than their controls.

In a recent study, Miledi (1960a) concluded that the innervated end-plate regions of partially denervated frog sartorius muscle fibres were not more sensitive to acetylcholine than the end-plate regions of innervated muscle fibres. However, he did not make this comparison experimentally, but based his conclusion on the observation that the responses recorded in the region of innervated end-plates of partially denervated and innervated muscles were not very different following acetylcholine administration. Without comparing the sensitivity of matched pairs of sartorius muscles to drug stimulation it would often be difficult and at times impossible to detect an increased sensitivity following either partial or complete denervation.

More difficult to explain is the marked difference in sensitivity to acetylcholine stimulation of the innervated and denervated end-plate regions of partially denervated muscle fibres observed by Miledi (1960a). Frank (1959) did not find this difference in tests carried out in the presence of neostigmine. Since at times the complete nerve supply to the denervated end-plate region was not sectioned and since the muscles were not examined for evidence of regeneration, it is possible that in the experiments of Miledi (1960a) regeneration of the nerve supply to the denervated end-plate region had occurred. If this was so, the innervated end-plate region would no longer be supersensitive to acetylcholine stimulation (Frank, 1959), but the denervated end-plates would maintain their increased sensitivity for a considerable period of time (Miledi, 1960b). Thus it is possible that under the conditions of the experiments of Miledi (1960a) the innervated end-plate regions of the partially denervated muscles did not have an increased sensitivity to acetylcholine, but this

does not mean that the innervated end-plates cannot develop this supersensitivity under the appropriate conditions.

In 1943 Kuffler reported that the non-innervated areas of frog sartorius muscle fibres as well as their end-plate regions develop an increased sensitivity to acetylcholine following complete denervation of the muscle. However, he emphasized the fact that the end-plate regions were still considerably more sensitive. Recently this increase in the area of membrane sensitive to acetylcholine following denervation has been more clearly demonstrated by the iontophoretic application of acetylcholine to minute areas of membrane in denervated mammalian skeletal muscle (Axelsson & Thesleff, 1959) and in denervated frog skeletal muscle (Miledi, 1960a). These investigators have put forward the hypothesis that this increase in the sensitive area of membrane is the change responsible for the increased sensitivity to applied drugs which follows denervation.

Two main lines of experimental evidence are used to support this hypothesis (Miledi, 1960a ; Axelsson & Thesleff, 1959 ; Thesleff, 1960). First is the demonstration itself of an enlarged area of increased sensitivity. Second, and most important, neither group observed an increased response to iontophoretically applied acetylcholine of the denervated end-plate region itself but only an increased response of the surrounding areas of membrane. In fact, in denervated mammalian skeletal muscle, the entire membrane appeared to have become uniformly sensitive to acetylcholine. However, the above workers neither compared dose-response relationships nor acetylcholine thresholds in innervated and denervated end-plates.

There is considerable evidence that the cholinesterase activity in the end-plate area decreases following denervation (Thesleff, 1960 ; Strömblad, 1960), which results in an increased response to applied acetylcholine (Thesleff, 1960 ; Axelsson & Thesleff, 1959 ; Frank, 1959). Since the responses to iontophoretically applied acetylcholine, when tested in the absence of an anticholinesterase, were no greater in the region of a denervated end-plate than in the region of an innervated end-plate (Axelsson & Thesleff, 1959 ; Miledi, 1960a), this would indicate that the acetylcholine doses were producing maximal responses. It is known that the maximal response of a skeletal muscle does not increase following denervation (Dale & Gasser, 1926 ; Cannon & Rosenblueth, 1949). Thus the responses to threshold or submaximal drug doses must be tested before rejecting the possibility that denervation results in an increased sensitivity of the end-plate areas to applied acetylcholine. Furthermore, it would be difficult to explain many of the results obtained in the present study as well as the results obtained by Dale & Gasser (1926) if it is assumed that there is no true increased sensitivity to applied drugs following denervation, but only an increased area of membrane sensitive to stimulation.

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PHARMACOLOGY OF METHICILLIN

BY

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The pharmacology of a new antibiotic methicillin, 6(2:6-dimethoxybenzamido)-penicillanic acid, which is effective against staphylococci resistant to penicillin G, has been investigated. It is free from acute and chronic toxic effects, except that some pain may be caused following intramuscular injection. It is poorly absorbed orally, but after intramuscular injection the concentrations in the serum and in tissues are very similar to those found with penicillin G. It is excreted by the kidneys both by renal tubular secretion and glomerular filtration. It is also excreted in the bile in very high concentrations, the ratio of concentration in the bile to the blood being approximately 2.5 times that of penicillin G. From a study of the metabolism of the drug it is calculated that 75% is eliminated unchanged in the urine and that the remainder is probably destroyed after the excretion into the intestine via the bile.

With the isolation of 6-aminopenicillanic acid (Batchelor, Doyle, Nayler & Rolinson, 1959), it is now possible to synthesize a wide range of new penicillins having modified antibacterial properties. One of these new derivatives, 6-(2:6-dimethoxybenzamido)penicillanic acid, has been shown to possess remarkable stability to staphylococcal penicillinase, and recent clinical reports have confirmed its effectiveness in the treatment of infections caused by staphylococci resistant to penicillin G (Douthwaite & Trafford, 1960; Knox, 1960; Stewart, Harrison & Holt, 1960; and Stewart, Nixon, Coles, Kesson, Lawson, Thomas, Mishra, Mitchell, Semmens & Wade, 1960). Preliminary pharmacological and chemotherapeutic findings have been reported by Brown & Acred (1960). Full details of the pharmacological evaluation are now presented.

METHODS

In all experiments penicillin G and methicillin were administered as their pure sodium salts. The antibiotics were assayed by the cup-plate technique using *Sarcina lutea* as the test organism. The zone diameters obtained for the control dilutions of the antibiotics were plotted against the log of the concentration, and from the regression line obtained the concentrations of the antibiotics in the specimens were estimated by interpolation. The appropriate dilutions of the controls and samples were made in phosphate buffer *p*H 7.0 (M/20) except in the experiments where serum concentrations were determined, in which case the controls were prepared in serum.

Acute toxicities

The acute toxicity was determined in male albino mice (18 to 22 g) after intravenous, subcutaneous and oral administration, and subcutaneously in rats (150 to 200 g). The effect on respiration and the electrocardiogram was studied in guinea-pigs and cats during the slow intravenous infusion of the antibiotic.

Prolonged administration

The effects of prolonged administration were investigated in rats and dogs.

(i) *Rats.* Two groups of 12 male rats, each animal weighing 80 to 100 g, were injected subcutaneously 5 days per week with methicillin at doses of 100 and 500 m_b/kg in a volume of 0.1 ml./100 g body weight for a period of 12 weeks. A group receiving 0.1 ml./100 g body weight normal saline subcutaneously acted as controls. Daily food intake and the weight of each rat was recorded. Weekly records of the red and white blood cell counts and qualitative tests for sugar and protein in the urine were performed. Haemoglobin determinations and spectroscopic examinations of the blood were made on the first, sixth and twelfth week of the test.

At the end of 6 weeks, 6 rats from each group were killed and the remaining rats killed at 12 weeks. The weights of the livers, spleens, kidneys, testes and adrenals were recorded, and specimens of liver, spleen, kidney, lung, thyroid, heart, duodenum, stomach, pancreas, adrenal, testis and bone marrow were removed for histological examination.

(ii) *Dogs.* Methicillin (250 mg/kg) was administered subcutaneously twice daily for a period of 4 weeks to two dogs. The following biochemical and haematological estimations were made at weekly intervals: haemoglobin (g%), packed cell volume, total white cell count, blood urea, serum alkaline phosphatase, zinc sulphate turbidity and serum globulin and albumin. A differential blood cell count was carried out at the end of the first and final week of the test.

Local irritant action

Ten per cent. and 1% solutions were injected intramuscularly and intradermally into rats and guinea-pigs (3 per group). The solutions were administered in a volume of 0.1 ml. intramuscularly into the hind legs, and 0.05 ml. intradermally on a shaved area on the backs. After 24 hr the area of the injection was examined and the skin and subcutaneous tissues removed for histological examination.

The effect of methicillin on the eye was examined in a group of 3 rabbits. A 1% solution in normal saline was dropped into a pocket formed by pulling out the lower left eyelid. The solution was held over the eye for 1 min. Saline was similarly applied to the right eye. The eyes were examined at 1, 2, 4, 8 and 24 hr afterwards for signs of irritation.

Blood pressure and respiratory effects

The carotid blood pressure of 5 cats anaesthetized with a 4% urethane/1% chloralose mixture (5 ml./kg intravenously) was recorded manometrically on a smoked drum. Respiration was recorded by a lever connected by means of a thread which was sewn to the skin over the xiphisternum. Methicillin in physiological saline was administered intravenously through the femoral vein at intervals of 5 min.

Absorption

(a) *Oral*

(i) *Rabbits.* 100 and 500 mg/kg doses of methicillin were administered orally to groups of 5 rabbits. Blood samples for assay were removed from the lateral ear vein at 1, 2, 4 and 6 hr.

(ii) *Absorption from small intestine of rats.* The bile ducts of rats, 5 to a group, were cannulated as described by Harrison *et al.* (1960). The rats were restrained in close-fitting wire cages and the total bile and urine collected at 2, 4, 6 and 24 hr after the administration of 100 mg/kg methicillin directly into the duodenum. Blood specimens were taken from the tail vein at 1, 3, 5 and 24 hr after injection.

(b) *Intramuscular—serum and urine concentrations in dogs*

Methicillin and penicillin G were injected intramuscularly at a dose of 5 mg/kg, and blood and urine specimens were taken at intervals up to 6 hr after administration.

The blood specimens were removed by means of a sterile syringe from the radial vein, allowed to clot at room temperature and the serum transferred to sterile tubes and frozen. The urine was removed from the bladder, by means of a polythene cannula, at the same time as the blood samples were taken.

Distribution and elimination

(a) *Serum and cerebrospinal fluid concentrations in rabbits*

(i) *Serum concentrations.* Two groups of 5 rabbits were given intramuscular injections, in the hind leg, of 100 mg/kg methicillin and 100 mg/kg penicillin G ; 0.5 ml. samples of blood were removed from the lateral ear veins at 1, 2, 4 and 6 hr after administration. The samples were allowed to clot at room temperature and the serum was removed and kept at 4° C until assayed (at the end of 6 hr).

(ii) *Cerebrospinal fluid concentrations.* The cerebrospinal fluid concentrations were determined in anaesthetized rabbits after administration of 100 and 500 mg/kg methicillin intramuscularly. The rabbits were anaesthetized with urethane (1 g/kg intraperitoneally). The cerebrospinal fluid samples were withdrawn from the cisternum magnum by means of a sterile syringe and needle. Blood samples were taken from the lateral ear vein at the time of taking the cerebrospinal fluid samples. The concentrations of methicillin in the serum and the cerebrospinal fluid were assayed as before.

(b) *Blood levels and bile excretion in the anaesthetized rat*

Two groups of 5 rats (230 to 265 g) were anaesthetized with 1.0 ml./kg pentobarbitone intraperitoneally. After laparotomy the bile duct was cannulated with polythene tubing (0.4 mm internal diameter). 100 mg/kg methicillin and penicillin G was administered intramuscularly to each of the groups. The bile was collected over 30 min periods in sterile tubes which were cooled in iced water. 0.1 ml. samples of arterial blood were obtained at hourly intervals up to 5 hr from the right carotid artery and diluted with 0.4 ml. normal saline containing 100 units heparin.

(c) *Hen*

The technique described by Sperber (1949) was employed. The cloacal mucosae of 6-month-old laying Sussex hens were anaesthetized with a few drops of 2% cinchocaine hydrochloride in normal saline. A polythene funnel was held in position over each ureteral opening by stitching the mucosa to the free edge of a rubber washer which surrounded the head of the funnel. Short polythene tubes were inserted into the stems of the funnels and the urine from each kidney collected in sterile tubes which were cooled in iced water.

The antibiotics were dissolved in 2.0 ml. of 0.9% saline and injected into the muscles of the left leg. Probenecid, which blocks renal tubular secretion, was administered intravenously at a dose of 100 mg. The probenecid solution was prepared from 0.5 g tablets (Merck Sharpe & Dohme). Five tablets were powdered and triturated with 10 ml. 0.5 N sodium hydroxide and the *p*H adjusted to 7.4 with 1.0 N hydrochloric acid (2 to 3 ml.). The volume was made up to 100 ml. with 0.9% saline, giving a solution containing 25 mg probenecid per ml. Four ml. of this solution was injected into a wing vein 10 min prior to the administration of the antibiotic.

In order to increase urine flow, 100 ml. of warm tap water was given by crop tube at the commencement of the experiment. Samples of urine were taken for assay at 10 min intervals for the first hour after dosing and thereafter at hourly intervals up to 6 hr.

In the experiments 4 hens were employed, each of which received the following treatments on four separate occasions: (i) 100 mg methicillin ; (ii) 100 mg penicillin G ; (iii) 100 mg methicillin + 100 mg probenecid ; (iv) 100 mg penicillin G + 100 mg probenecid.

(d) *Distribution in tissues*

100 mg/kg methicillin was administered intramuscularly to groups of 10 rats, 1 group being killed at 0, ½, 1, 2, 4, 12 and 24 hr after the injection. The rats were exsanguinated by cutting the throat and the blood collected. The urine and faeces from each group were

collected and the amounts recorded. The following organs and tissues were removed and weighed: liver, spleen, kidney, lung, small intestine, large intestine and muscle at site of injection. The carcass was then weighed. All specimens were homogenized in a Waring blender. Appropriate dilutions of the homogenate were made with phosphate buffer *p*H 7.0 and specimens of the homogenate assayed. The total amount of antibiotic in each specimen was calculated. The specimens which were known to be contaminated were not specially treated, as we found that in general the assay figures obtained were not unduly influenced.

Serum binding—dialysis

Five ml. of bovine, horse or human serum containing 5 mg of either methicillin or penicillin G was placed in cellophane bags (Visking tubing ¾ in.) and suspended in 20 ml. sterile saline at 10° C for 48 hr. At the end of the period of dialysis the amounts of the antibiotic outside and inside the tubing were assayed. Five tubes were prepared of each antibiotic in each experiment.

RESULTS

Toxicities

(i) *Acute*

Methicillin when given intravenously to mice at doses up to 2.5 g/kg produces no signs of toxicity. When larger doses are given, 3 to 5 g/kg, occasional mild clonic convulsions are produced within 5 min of administration. These give way to a phase which may persist up to 4 hr in which the mice exhibit a loss of muscle tone. If disturbed during this phase, a mild muscular spasm is induced causing extension of the limbs. In the range 4 to 5 g/kg some deaths may occur due to cardiac and respiratory failure. With some animals respiration may cease for periods up to 10 sec, but this is followed by complete recovery. No toxic symptoms were noted in mice and rats given 4 g/kg of the antibiotic orally or subcutaneously.

(ii) *Prolonged administration*

(a) *Rats.* No untoward toxic symptoms were noted in the rats treated with methicillin. Post-mortem examination did not reveal any abnormalities and the

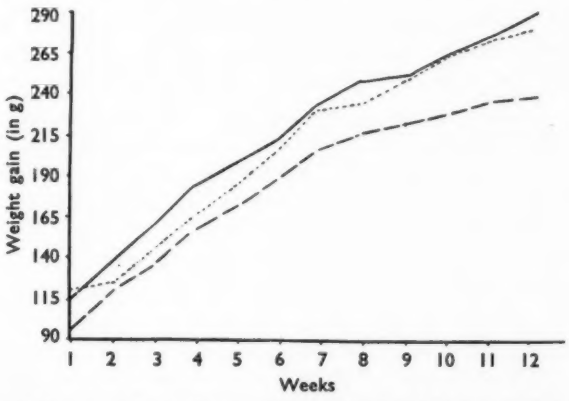

Fig. 1. Mean growth curves of rats—12 to a group—following daily intramuscular administration of (*a*) 500 mg/kg methicillin (——); (*b*) 100 mg/kg methicillin (- - - -); (*c*) 1.0 ml./kg normal saline (— — —).

histology of the organs examined was normal. The growth curves are shown in Fig. 1. The animals which received the antibiotic gained more weight than the controls.

(b) *Dogs.* No abnormal signs were observed in any of the tests carried out. The histology of the essential organs was normal.

Local irritant action

Macroscopically there was no evidence of tissue damage, except following the 10% intradermal injection, when there was a slight induration and erythema of the skin lasting 24 to 48 hr. Histologically there is a marked inflammatory reaction in the rat skin with necrosis of epidermis and hair follicles, following intradermal injection of 10% methicillin. A comparable inflammatory reaction is seen in the muscle. The changes in both skin and muscle of rats are much less marked with the 1% solution. The changes in the guinea-pig skin and muscle are similar; as severe in the skin at 10% but less marked in the muscle at that strength. The skin and muscle of guinea-pigs injected with the 1% solution showed no appreciable abnormality.

Cardiovascular and respiratory effects

When administered by slow intravenous injection to 5 guinea-pigs, 5 g/kg methicillin in saline given over a period of 25 min had no observable effect on the respiration or the electrocardiogram. When similarly administered to cats, 2.5 g/kg given over a 10 min period had no effect on the heart or respiration. In individual doses ranging from 10 to 500 mg/kg, methicillin was again without effect. The blood pressure response of cats to intravenous doses of adrenaline, noradrenaline, acetylcholine and histamine was unaffected by doses of 500 mg/kg methicillin. The pressor response to carotid occlusion was also unaffected by methicillin.

Absorption

(a) *Oral*

(i) *Rabbits.* No blood levels were detectable after 100 mg/kg, but significant blood levels were attained following administration of 500 mg/kg, viz., 3.0, 1.6, 1.6 and 1.45 μg/ml. at 1, 2, 4 and 6 hr respectively after administration.

(ii) *Absorption from small intestine of rats.* The concentration of methicillin found in the bile, urine and the blood after the intraduodenal administration of 100 mg/kg to rats is given in Table 1. Practically no blood levels were detectable, and only a total of 6.5% of the antibiotic was recovered—1.8% from the bile, 3.7% from the urine and 0.97% from the intestine.

(b) *Intramuscular—serum and urine concentrations in dogs*

The mean serum concentrations of penicillin G and methicillin obtained in three dogs after intramuscular injection of 5 mg/kg are shown in Fig. 2. Methicillin gives a slightly lower maximum blood concentration (2.9 μg/ml.) than penicillin G (4.05 μg/ml.). The peak also occurs slightly more in advance of penicillin G, but the fall-off in serum concentration of both antibiotics occurs at much the same rate. The excretion in the urine of both antibiotics is also very similar, 29% of penicillin G and 33% of methicillin being recovered in the urine in 6 hr.

TABLE 1

ABSORPTION OF METHICILLIN IN THE CONSCIOUS RAT AFTER INTRADUODENAL DOSING WITH 100 MG/KG

Rat	Dose in mg	Bile: % of dose excreted (hr) 0–2	2–4	4–6	6–24	Urine: % of dose excreted (hr) 0–2	2–4	4–6	6–24	Blood levels: concentration μg/ml. (hr) 1	3	5	24	% of dose remaining in intestine after 24 hr	Total % recovered
B	30	0·12	0·12	0·17	0·23	1·30	0·60	0·16	2·45	0·28	0·39	0	0	3·40	8·55
D	34	1·02	0·31	0·42	0·86	2·06	0·69	0·29	1·87	0	0	0	0	0·74	8·26
E	27	0·57	0·29	0·27	0·12	1·04	0·79	0·41	0·51	0	0	0	0	0	4·00
F	26	0·12	0·63	0·42	0·48	0·39	0·95	0·85	1·88	0	0	0	0	0·71	6·43
G	28	0·64	0·75	0·56	1·14	0·93	0·24	0·42	0·70	0	0	0	0	0	5·38
Mean % of dose excreted		0·49	0·42	0·36	0·56	1·14	0·66	0·42	1·48					0·97	6·52
Cumulative % excreted		0·49	0·91	1·27	1·83	1·14	1·80	2·22	3·70						

TABLE 2

DISTRIBUTION OF METHICILLIN IN THE RAT

Seven groups of 10 rats were injected intramuscularly with 100 mg/kg methicillin. One group was killed at the end of each time period. The mean concentration of methicillin is expressed in μg/g wet weight of tissue (column a) and the concentration ratio between the tissues μg/g wet weight to serum μg/ml. is shown in column b. The urinary excretion is expressed as a % of the dose administered

	0 hr		0·5 hr		1 hr		2 hr		4 hr		12 hr		24 hr	
	a	b	a	b	a	b	a	b	a	b	a	b	a	b
Liver			32·8	1·01	23·8	0·79	12·9	2·36	4·1	22·8	0	—	0	—
Spleen			4·5	0·14	2·03	0·07	1·47	0·27	0	0	0·68	—	0	—
Kidneys			122·1	3·76	88·8	2·94	24·1	4·41	2·91	16·17	0·22	—	0	—
Lungs			15·6	0·42	8·8	0·29	2·34	0·43	1·36	7·56	0·19	—	0	—
Small intestine			74·9	2·3	133·9	4·43	149·6	27·4	54·7	304·0	1·05	—	0	—
Large intestine			5·31	0·16	6·84	0·23	7·76	1·42	31·41	14·45	14·5	—	0	—
Injection site	1,620·0		821·0	25·27	332·5	11·01	74·65	13·67	7·8	43·34	0·64	—	0	—
Carcass	2·0		11·8	0·29	9·39	0·31	2·52	0·46	3·7	20·6	0·71	—	0	—
Serum			32·5	1·0	30·2	1·0	5·46	1·0	0·18	1·0	0	—	0	—
Urine % excreted			13·39		32·6		42·8		48·9		74·6		68·4	

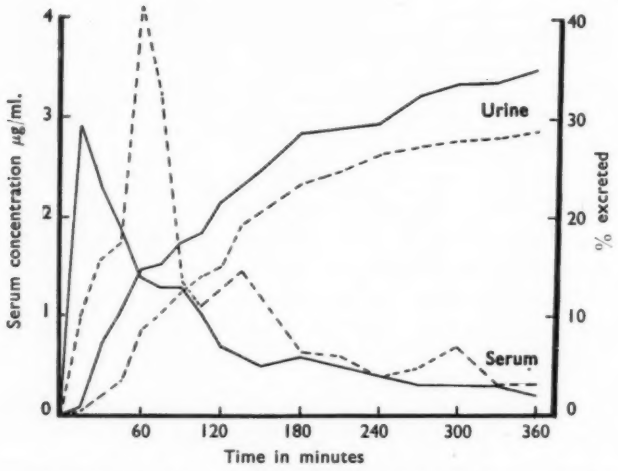

Fig. 2. Serum concentration and urinary excretion in dogs following intramuscular administration of 5 mg/kg methicillin (——) and penicillin G (– – –).

Distribution and elimination

(a) *Serum and cerebrospinal fluid concentrations in rabbits*

(i) *Serum concentrations.* The serum concentrations of penicillin G and methicillin after intramuscular injection to each of 5 rabbits are given in Table 3. The serum concentration picture of each antibiotic is similar.

TABLE 3

SERUM CONCENTRATIONS IN RABBITS RECEIVING 100 MG/KG METHICILLIN AND PENICILLIN G INTRAMUSCULARLY

	Serum concentration μg/ml. at hr after dosing			
Compound	1	2	4	6
Methicillin	12·5	3·65	0·51	0
	18·0	3·65	0·3	0
	14·0	4·2	0·74	0
	18·0	5·0	0·34	0
	14·0	6·2	1·3	0
Mean	15·3	4·48	0·64	0
Penicillin G	11·0	5·1	2·65	1·13
	9·0	5·0	1·17	0·5
	16·0	10·5	1·0	0·1
	10·5	4·7	0·48	0
	9·8	6·1	1·15	0·23
Mean	11·26	6·28	1·29	0·39

(ii) *Cerebrospinal fluid concentration.* The concentration of methicillin obtained in the serum and cerebrospinal fluid after the intramuscular administration of 100 and 500 mg/kg is shown in Table 4. The concentration of antibiotic in the cerebrospinal fluid is approximately 100 times less that the concentration appearing in the

TABLE 4

MEAN CONCENTRATIONS IN SERUM AND CEREBROSPINAL FLUID OF RABBITS (3 PER DOSE LEVEL) AFTER INTRAMUSCULAR ADMINISTRATION OF METHICILLIN

Dose mg/kg	Tissue	Concentration μg/ml. at hr after dosing 0·5	1	2	3	6
100	C.s.f.	0·4	0·35	—	—	0
	Serum	34·0	24·0	—	—	0
500	C.s.f.	1·17	2·3	3·6	4·4	0
	Serum	130	136	288	130	1·0

serum. The time of maximum concentration of methicillin in the serum does not coincide, however, with the time of maximum concentration in the cerebrospinal fluid, the latter occurring somewhat later.

(b) *Blood levels and bile excretion in the anaesthetized rat*

The blood picture of the anaesthetized rat after the administration of penicillin G and methicillin is shown in Fig. 3. The blood levels of both antibiotics are similar and both are markedly concentrated in the bile, though at the end of 6 hours both the blood and bile levels of methicillin were a little higher than those of penicillin G.

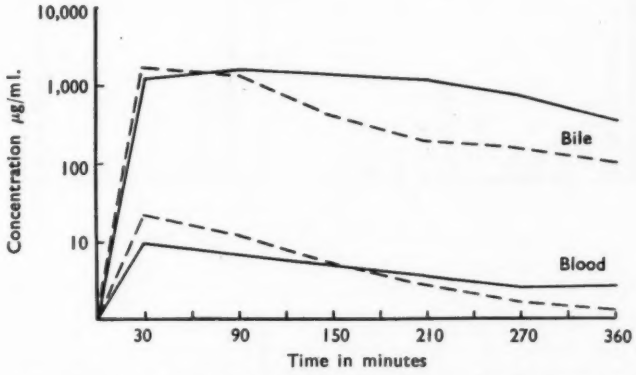

Fig. 3. Mean concentration in blood and bile of methicillin (——) and penicillin G (– – –) after intramuscular administration of 100 mg/kg of each antibiotic to groups of 5 anaesthetized rats.

(c) *Hen*

The excretion in urine of penicillin G and methicillin from the right and left kidneys following intramuscular administration into the left limb of 100 mg/kg of each antibiotic is illustrated in Fig. 4. The concentration of both antibiotics in the urine from the left kidney is considerably greater than the concentration in the urine from the right kidney. However, after the administration of 100 mg probenecid intravenously the concentration of both antibiotics in the urine from the right and left kidneys becomes identical.

(d) *Distribution in tissues*

The tissue concentrations of methicillin expressed as μg/g of wet weight of tissue are given in Table 2 (column a). In order to show the degree of concentration in the

various tissues and organs, the results have also been expressed as a ratio of the serum concentration (column b). Thirty min after the dose was given, the concentration occurring in the kidneys was approximately four times, that in the small intestine about double, and that in the liver about the same as that found in the serum. Lower concentrations occurred in the lungs, spleen and large intestine. The concentration of antibiotic in the serum falls off rapidly, but the concentration level in the other tissues apart from the spleen falls off more slowly, with the result that at the end of 4 hr the concentrations in the tissues are higher than those found in the serum. At the end of 12 hr there are only trace amounts left in most of the tissues,

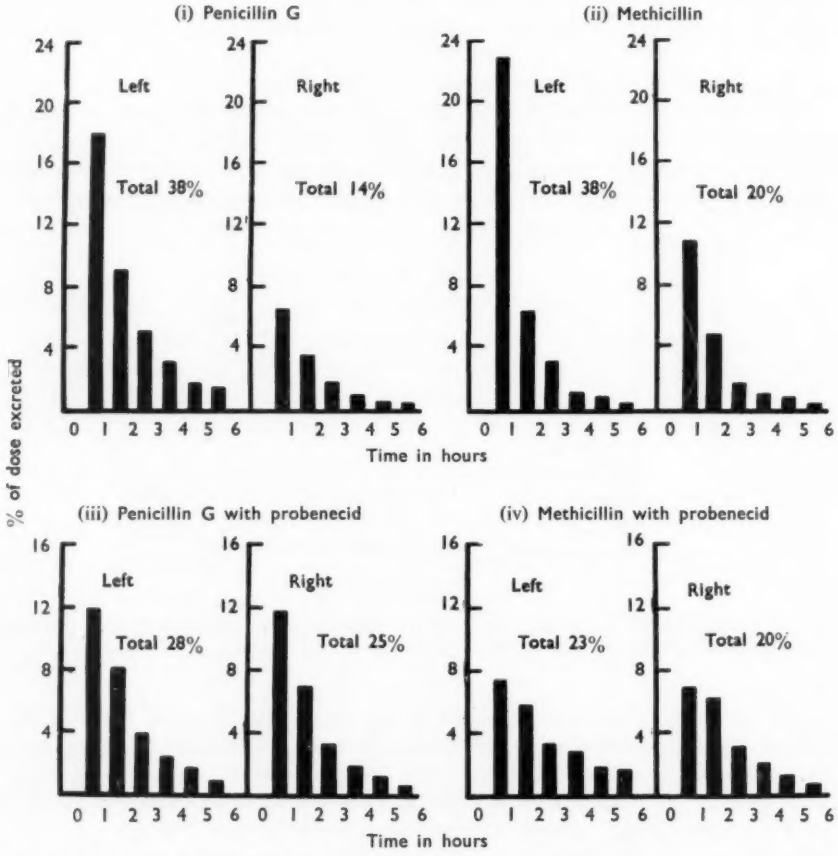

Fig. 4. Excretion of methicillin and penicillin G in the hen. The mean urinary excretion from the left and right kidneys of 4 hens each of which received, on four separate occasions: (i) 100 mg penicillin G; (ii) 100 mg methicillin; (iii) 100 mg penicillin G+100 mg probenecid; (iv) 100 mg methicillin+100 mg probenecid. The antibiotics were given intramuscularly and the probenecid intravenously.

none being detectable in the serum and liver. After 24 hr no antibiotic was detectable in any of the tissues. The urinary excretion is expressed as a percentage of the dose administered, a total of 74.6% being recovered in the first 12 hr. A total of 73.7%, 73.5%, 66.6%, 55.3%, 54.3%, 75.1% and 68.4% of methicillin was recovered from all tissues and body fluids at 0, 0.5, 1, 2, 4, 12 and 24 hr after administration.

Serum binding

Methicillin is considerably less bound to horse, bovine and human serum than penicillin G, the figures being respectively for each of the sera 10.2%, 4.5% and 17.7% for methicillin, and 21.0%, 27.3% and 43.4% for penicillin G.

DISCUSSION

An antibiotic should preferably be free from toxic effects. In this respect methicillin has remarkably few unwanted reactions. No toxic effects were noted in animals after large single intravenous doses. In one experiment a dose of 18 g was administered by slow intravenous infusion to a guinea-pig before serious toxic effects became apparent. When administered over a prolonged period both rats and dogs tolerated large doses, the only observable reaction being some discomfort at the time of injection. This is in agreement with clinical reports (Douthwaite & Trafford, 1960 ; Knudsen & Rolinson, 1960).

Methicillin is poorly absorbed from the gastro-intestinal tract, but it is well absorbed following parenteral administration, only small quantities remaining at the site of injection 12 hr afterwards. Good blood levels are obtained, and the antibiotic is distributed throughout the tissues of the body. There is a high concentration of antibiotic in the kidney, while the concentration in the liver is of the same order as that found in the serum for the first hour, but higher concentrations than those in the serum are subsequently found. The high concentrations appearing in the small intestine are probably accounted for by the presence of bile.

The total quantity of methicillin recovered from the intact animal falls steadily over the first 4 hr to 55%, but fully 75% is eventually recovered in the urine after 12 hr. It would seem that the antibiotic is taken up by some of the tissues in a non-assayable form and is then slowly released and then excreted in the urine.

That methicillin does combine with protein is shown by the dialysis experiments. There is considerable binding with serum protein, but it is less than that found with penicillin G. The degree of binding varies according to species, and it is possible that some of the antibiotic is more firmly bound to one type of protein than another and therefore it is more difficult to assay in this state. The antibiotic may be slowly released from this combination. Otherwise, it is difficult to postulate why there is a " loss " which is subsequently recovered in the urine.

The blood level picture and urinary excretion found for methicillin and penicillin G are practically identical, the pattern being similar in rabbits, dogs and conscious rats. In the anaesthetized rat the excretion is delayed, 50 to 55% of the antibiotics appearing in the urine of conscious rats during a 4 hr period, while only 25 to 35% is recovered in 6 hr from an anaesthetized animal, and as a consequence the blood

level is more prolonged. In view of this difference it would seem preferable to use non-anaesthetized animals for metabolism studies.

A marked difference is seen between the bile concentrations of methicillin and penicillin G. Comparison of the bile/blood ratio for the two antibiotics obtained in rats shows that methicillin is concentrated in the bile fully 2 to 2.5 times more than penicillin G. A total of 15 to 20% of the injected dose of methicillin is excreted in the bile—this we confirmed in both the anaesthetized and conscious rat—while 15% of penicillin G is similarly excreted. In view of the greater concentration ratio of methicillin from the blood to the bile, it might be expected that the difference between the amounts excreted would be more, but the initial blood levels of penicillin G are higher and thus proportionately a larger quantity appears in the bile.

In the normal animal one would expect that a considerable proportion of the antibiotic would be reabsorbed from the intestine after being excreted in the bile. However, the experiments where 100 mg/kg was injected into the duodenum of rats indicate that absorption even in the small intestine is poor—only 3.7% is recovered from the urine and 1.8% from the bile in 24 hr. The bulk of the dose appears to be destroyed, as only 0.97% can be accounted for in the intestine after 24 hr. From the observations on the distribution of the antibiotic in the intact animal one can therefore account for practically the whole of the dose administered, 75% being excreted in the urine and 15% lost in the intestine. We have found no evidence that 6-aminopenicillanic acid is formed in the body from methicillin; neither have we found that methicillin is excreted in a conjugated form in the bile as reported by Harrison *et al.* (1960).

The mode of elimination by the kidneys is well demonstrated in the hen experiment. Birds possess a renal portal system, and the venous return from the hind limbs is shunted through the parenchyma of the renal tubules. Therefore, when a substance is injected into a hind limb and both renal tubular secretion and glomerular filtration take place, the concentration of the substance excreted is greater in the urine from the ipsilateral kidney than in the urine from the contralateral kidney. If only glomerular filtration takes place, then the concentration in the urine excreted from both kidneys is identical. In our experiments the ipsilateral kidneys excreted more of the antibiotics than the contralateral kidneys, and blockage of renal tubular secretion resulted in equal concentrations of the antibiotics appearing in the urine from both kidneys. Therefore methicillin and penicillin G are excreted by the same mechanism, both glomerular filtration and renal tubular secretion taking place.

We wish to thank Dr A. C. Thackray, of the Bland Sutton Institute of Pathology, for expert histological comment; Dr A. A. G. Lewis, physician to the Connaught Hospital, Walthamstow, under whose skilled guidance the dog studies were conducted; and Mr F. P. Doyle and his colleagues for the preparation of the compound.

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ACTION OF BRETYLIUM ON THE ISOLATED GUINEA-PIG ILEUM

BY

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Bretylium inhibits the contractions of the longitudinal muscle caused by histamine and, to a lesser extent, those by acetylcholine, carbachol and nicotine. It is a very weak antagonist of 5-hydroxytryptamine and in concentrations of up to 190 μg/ml. does not inhibit the actions of bradykinin and substance P on the longitudinal muscle. The inhibitory effect of bretylium on the emptying phase of the peristaltic reflex is similar to that of ganglion blocking agents, while the much less pronounced effect on the contraction of the longitudinal muscle during the preparatory phase is assumed to be due to a weak atropine-like action.

When Boura & Green (1959) reported their experiments on the inhibition of adrenergic neurones by bretylium, they described a number of other effects. One of these, the inhibitory effect on the peristaltic reflex in the isolated guinea-pig ileum, attracted our attention, as we thought that it might yield information on the nature of this reflex. Since adrenaline and noradrenaline depress the peristaltic reflex (Kosterlitz & Robinson, 1957), its inhibition by bretylium cannot be due to a block of adrenergic neurones.

Boura & Green (1959) also observed that the contractions of the longitudinal muscle of the ileum caused by histamine were reduced by bretylium but not those caused by acetylcholine or 5-hydroxytryptamine. These findings have been re-examined by us and extended to the contractions of the longitudinal muscle produced by other substances.

METHODS

The method of Trendelenburg (1917) was used for investigating the effect of bretylium on the peristaltic reflex of the isolated guinea-pig ileum. Contractions of the longitudinal muscle layer caused by addition of drugs to the bath fluid were recorded either isotonically by a conventional light lever or isometrically by a mechano-electrical transducer (Innes, Kosterlitz & Robinson, 1957).

The bath fluid was Tyrode solution with a low magnesium sulphate content (0.01 g/l.), oxygenated with 100% O_2. There were intervals of 3 min between the individual experimental procedures such as the eliciting of the reflex or the addition of drugs. The bath fluid was renewed at least every 6 min to prevent a significant rise of the *p*H above the initial value of about 8.

A segment of the distal end of the guinea-pig ileum was used after discarding the 10 cm nearest to the ileo-caecal valve. The ileum was set up with an initial tension of 1 *g* about 1 hr before the beginning of the experiment.

The doses of the drugs are given in weight of base for acetylcholine chloride, carbachol (carbaminoyl-choline chloride), histamine diphosphate, 5-hydroxytryptamine creatinine sulphate and nicotine bitartrate; in weight of salt for hexamethonium iodide and bretylium tosylate. Substance P and bradykinin were used as partly purified preparations; the doses of the latter are given in Roche e Silva units.

In confirmation of the findings of Boura & Green (1959), no difference was observed between the actions of bretylium tosylate and bretylium bromide.

RESULTS

Effect of bretylium on contractions of the longitudinal muscle caused by drugs

Acetylcholine, carbachol and histamine. Bretylium depressed the responses of the longitudinal muscle to all three substances, the minimum effective concentration being 2 μg/ml. The inhibitory effect, which reduced both the height and the speed of contraction, was observed within 3 min of the addition of bretylium to the bath; it was readily reversible by washing out the drug.

Bretylium antagonized the action of histamine more powerfully than that of acetylcholine (Fig. 1). When the ratios of the doses causing equal contractions in

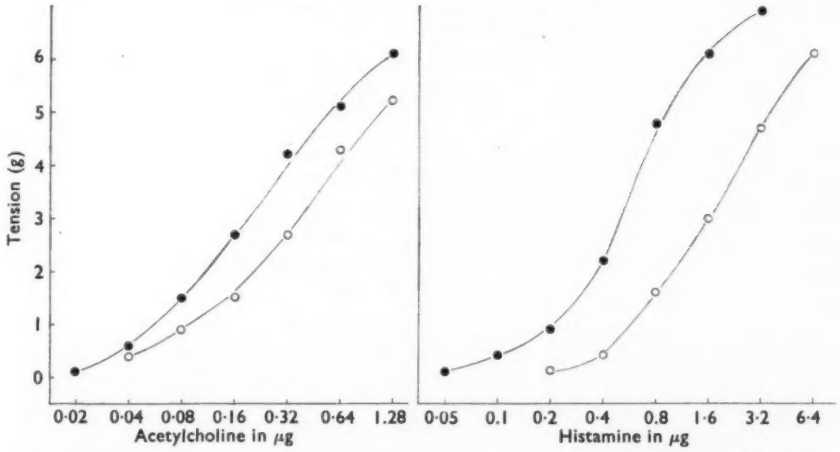

Fig. 1. Dose-response curves for acetylcholine and histamine alone (●——●) and in the presence of bretylium tosylate 14 μg/ml. bath fluid (○ —— ○). Abscissa: doses of acetylcholine or histamine added to 40 ml. bath. Ordinate: tension developed in isometric contractions of the longitudinal muscle.

the presence (14 μg/ml.) and absence of bretylium were calculated, they were found to be 4.3 in the case of histamine and 2 in the case of acetylcholine.

5-Hydroxytryptamine and nicotine. Drugs which cause contraction of the longitudinal muscle by stimulation of neuronal elements were also antagonized by bretylium. In a concentration of 18 μg/ml., bretylium depressed the action of nicotine but not that of 5-hydroxytryptamine. The smallest concentration of bretylium which had an inhibitory effect on the contraction due to 5-hydroxytrypt-

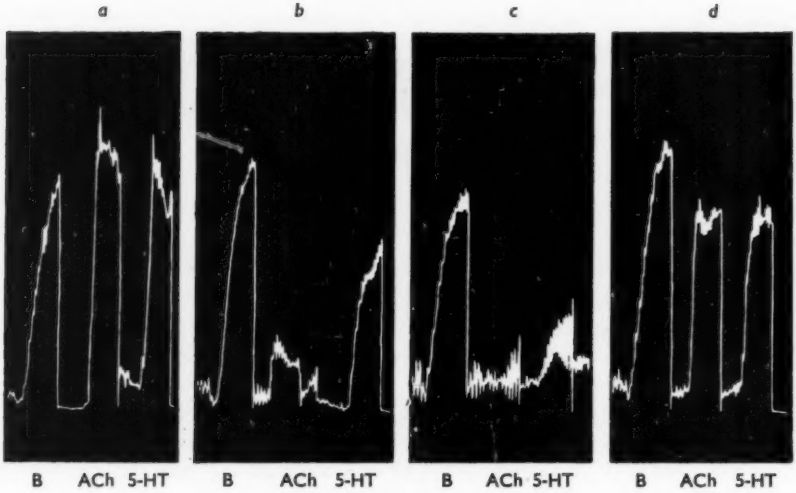

Fig. 2. The effect of bretylium on the contractions of the longitudinal muscle caused by bradykinin, acetylcholine and 5-hydroxytryptamine. Bath containing 7·5 ml. Tyrode solution. (*a*) Controls; (*b*) 5 min after addition of bretylium tosylate (100 μg/ml. bath fluid) ; (*c*) 14 min after increasing the bretylium concentration to 190 μg/ml.; (*d*) 12 min after washing out bretylium. B: 0·5 units bradykinin; ACh: 10 ng acetylcholine; 5-HT: 0.2 μg 5-hydroxytryptamine.

amine was 100 μg/ml. (Fig. 2). The ratio of doses of nicotine having equal potency in the presence (11 μg/ml.) and absence of bretylium was 1.5.

Bradykinin and substance P. The actions of these two polypeptides were not inhibited by bretylium in concentrations up to 190 μg/ml. In these large doses, bretylium itself caused a transient contraction of the longitudinal muscle, an observation already made by Boura & Green (1959). When the bretylium was left in the bath, the muscle relaxed again after 3 to 5 min and for a further period of about 10 min remained insensitive to the stimulating drugs used, an effect similar to that described by Cantoni & Eastman (1946). Thereafter the responses to the polypeptides returned to normal while those to acetylcholine, histamine and 5-hydroxytryptamine remained depressed. For instance, in the presence of 100 to 190 μg/ml. of bretylium, the contractions due to acetylcholine and 5-hydroxytryptamine were markedly reduced, while those caused by bradykinin were affected only a little, if at all (Fig. 2).

Effect of bretylium on the peristaltic reflex elicited by distension of the lumen

The inhibitory effect of bretylium was more marked on the second or emptying phase of the reflex than on the first or preparatory phase. The contractions of the circular muscle during the emptying phase were abolished by concentrations of 5 to 18 μg/ml., which had no effect on the contractions of the longitudinal muscle layer during the preparatory phase (Fig. 3).

These experiments were repeated after blocking the emptying phase with hexamethonium (25 μg/ml.). Again, bretylium in concentrations of 5 to 18 μg/ml. had

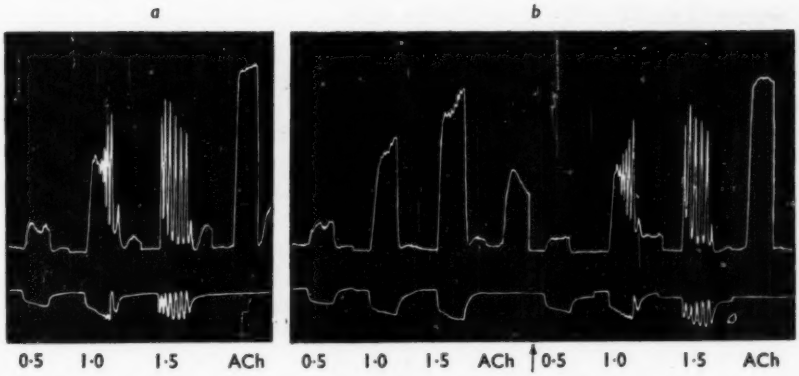

Fig. 3. The effect of bretylium on the peristaltic reflex. 40 ml. bath. Upper tracing: contraction of longitudinal muscle; lower tracing: filling of intestinal lumen. (*a*) Controls; (*b*) 15 min after addition of bretylium tosylate (15 μg/ml. bath fluid), washed out at arrow. The figures below the tracings indicate intra-intestinal pressures in cm of water. ACh: 0.1 μg acetylcholine.

no inhibitory effect on the contraction of the longitudinal muscle during the preparatory phase, although the responses to acetylcholine, carbachol and histamine were reduced. When the concentration of bretylium was raised beyond 100 μg/ml. the preparatory phase was partly inhibited, the contraction due to acetylcholine (1.3 ng/ml.) almost completely abolished while that due to bradykinin was scarcely affected (Fig. 4).

The inhibitory effect of bretylium on the emptying phase was observed within 3 min of the addition of the drug to the bath fluid ; recovery took place within 3 min after washing it out. With the larger doses required for inhibition of the longitudinal contraction during the preparatory phase, recovery was delayed for 20 to 30 min.

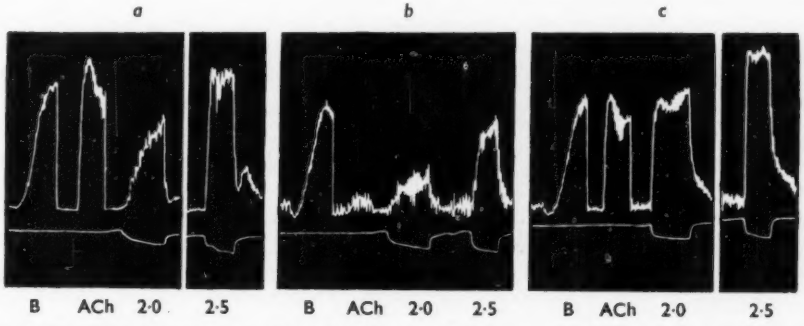

Fig. 4. The effect of bretylium on the peristaltic reflex in the presence of hexamethonium (25 μg/ml. bath fluid). Bath containing 7.5 ml. Tyrode solution. Upper tracing: contraction of longitudinal muscle; lower tracing: filling of intestinal lumen. (*a*) Controls; (*b*) 16 min after addition of bretylium tosylate (190 μg/ml. bath fluid); (*c*) 31 min after washing out bretylium. The figures below the tracings indicate intra-intestinal pressures in cm of water. B: 0.35 units bradykinin; ACh: 10 ng acetylcholine.

DISCUSSION

Our results confirm the findings of Boura & Green (1959) as far as the inhibitory effect of bretylium on the peristaltic reflex and on the contraction of the longitudinal muscle due to histamine is concerned. We find, in addition, that bretylium antagonizes, although to a lesser extent, acetylcholine, carbachol and nicotine, and that the inhibitory effect on the acetylcholine and carbachol contractions is also observed in the presence of hexamethonium. These findings suggest that bretylium has a weak atropine-like action.

The reversible inhibitory effect of low concentrations of bretylium (5 to 20 μg/ml.) on the peristaltic reflex is similar to that found with ganglion-blocking agents, in that it affects only the emptying phase and not the shortening of the gut during the preparatory phase (Feldberg & Lin, 1949; Paton & Zaimis, 1949; Kosterlitz & Robinson, 1957). The onset of the block of the cholinergic neurones is rapid and recovery is quickly brought about by washing out the drug. Boura & Green (1959) had already found that bretylium causes a readily reversible ganglionic block in the perfused superior cervical ganglion of the cat, and stressed that this is very different from the slow development and long persistence of the block which bretylium produces in adrenergic neurones.

The fact that high concentrations of bretylium (100 to 200 μg/ml.) inhibit the contractions of the longitudinal muscle during the preparatory phase is not necessarily in disagreement with the view that the inhibition of the emptying phase is due to a ganglion-blocking action. While the reflex arc of the preparatory phase does not contain cholinergic synapses, the transmission at the neuro-effector junction is inhibited by atropine (Schaumann, 1955; Kosterlitz & Robinson, 1957, 1959). It may therefore be assumed that the inhibition of the preparatory phase by high concentrations of bretylium is due to its weak atropine-like action.

We are indebted to Mr A. F. Green for supplies of bretylium bromide and bretylium tosylate, to Dr J. H. Gaddum for partly purified substance P, and to Dr M. Schachter for partly purified bradykinin.

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EFFECT OF RESERPINE ON THE 5-HYDROXYTRYPTAMINE AND ADENOSINETRIPHOSPHATE OF THE DOG INTESTINAL MUCOSA

BY

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(*Received March* 23, 1961)

Homogenates were prepared of the mucous membrane of the small intestine from dogs previously treated with high doses of reserpine. The granular material sedimented from these homogenates was centrifuged over a density gradient. The dense fraction usually rich in adenosinetriphosphate and 5-hydroxytryptamine contained little amine and no nucleotide; on the other hand, the less dense material, believed to consist mainly of mitochondria, contained amounts of adenosinetriphosphate similar to those found in animals not treated with reserpine.

Observations have recently been reported (Prusoff, 1960) which suggest that in the mucous membrane of the dog small intestine the 5-hydroxytryptamine present is associated with adenosinetriphosphate. The granular fraction containing the mitochondria as well as that containing the storage elements for 5-hydroxytryptamine was rich in nucleotide. The " large " granule sediment prepared from a homogenate of the tissue in isotonic (0.3 M) sucrose was resolved by ultracentrifugation over a sucrose density gradient and the distribution of both 5-hydroxytryptamine and adenosinetriphosphate was studied. The observations of Baker (1959) were confirmed, in which much of the amine was recovered not in the true mitochondrial fraction but in a particulate layer of greater density below the mitochondria. The distribution of the adenosinetriphosphate differed from that of the amine: both the mitochondrial layer and the dense layer rich in amine contained adenosinetriphosphate. In three experiments the mean molar ratio, 5-hydroxytryptamine: adenosinetriphosphate, in the dense layer was found to be 2.6; this is of an order similar to that of about 4 found in the chromaffin granules of the adrenal medulla (Blaschko, 1959). This finding suggested that in the storage of amine in the intestine the part played by the nucleotide was analogous to its role in the storage of the catechol amines in the chromaffin tissue.

To obtain further information on the relationship between adenosinetriphosphate and stored amines it was decided to investigate particulate fractions of mucosa from the small intestine of dogs in which the 5-hydroxytryptamine content had been greatly reduced by previous administration of reserpine. The results obtained are in support of a close association of the amine with adenosinetriphosphate in this tissue.

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METHODS

Reserpine dissolved in a 20% solution of ascorbic acid was administered intraperitoneally to dogs on three successive days in a dose of 0.5 mg/kg body weight. On the fourth day pentobarbitone sodium was given intraperitoneally and the dog was bled. The small intestine was quickly removed and a homogenate of the mucous membrane prepared as already described (Prusoff, 1960). Following the separation of unbroken cells, nuclei and course cell debris by centrifugation at 900 *g* for 20 min, the supernatant was spun at 11,000 *g* for 30 min in order to sediment the non-microsomal cytoplasmic particles. The sediment was resuspended in 0.3 M sucrose and layered over a sucrose density gradient prepared the previous day (Fig. 1). The tubes for the Spinco ultracentrifuge swing-out head (SW39) contained at the bottom 1.0 ml. 2.0 M sucrose, with 1.0 ml. 1.6 M sucrose layered over this, and on the top 1.5 ml. 1.5 M sucrose solution. The fractions, after ultracentrifugation for 1 hr at 100,000 *g*, were collected with the help of the Schuster cutter.

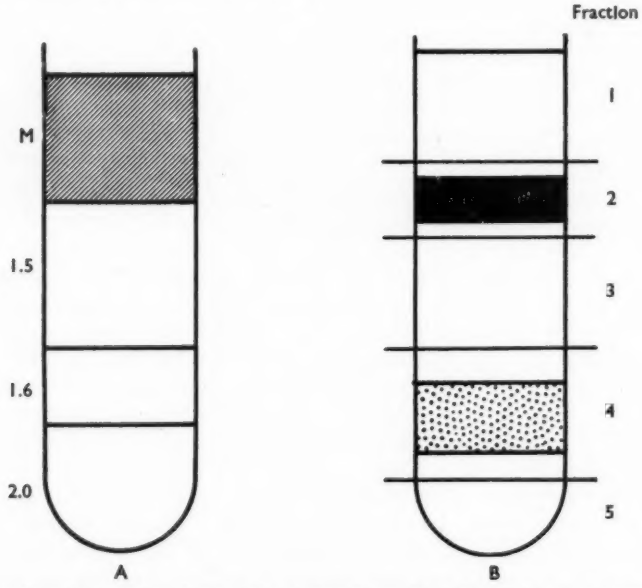

Fig. 1. Centrifugation of non-microsomal cytoplasmic particles obtained from the small intestine of dogs treated with reserpine. (A) Sucrose density gradient before centrifugation. (B) Distribution of particulates in density gradient after centrifugation for 60 min at 100,000 *g*.

5-Hydroxytryptamine was extracted with *n*-butanol by the method of Udenfriend, Weissbach & Clark (1955), as modified by Cargill-Thompson, Hardwick & Wiseman (1958) and Baker (1959), and assayed on the rat fundus preparation (Vane, 1957). Adenosinetriphosphate was estimated by the firefly luminescence method, as used by Holton (1959).

RESULTS

The two dogs on which these experiments were carried out showed the characteristic picture of animals treated with large doses of reserpine.

Although the appearance of the density gradient tubes after ultracentrifugation was essentially similar in both experiments, there were slight differences in the lower

half of the tube which determined slight variations in the choice of levels of cutting. In experiment no. 1 the appearance of the gradient tube was as shown in Fig. 1. There was a very dense and thick opaque layer at the top of the gradient (fraction 2); above this was a clear layer, corresponding to the vol. of fluid in which the suspension of large granules had been suspended before centrifugation (fraction 1). Below fraction 2 there was a relatively clear layer in which small amounts of opaque material were suspended; this was collected as fraction 3. Immediately below there was one well-defined, fairly narrow opaque layer in the 1.6 M sucrose layer (fraction 4). At the bottom of the tube, approximately corresponding to the 2.0 M sucrose layer, there was a clear area (fraction 5).

In the second experiment, fractions 1 and 2 each had the same appearance as in the first experiment, but below fraction 2 a slightly narrower clear zone (fraction 3) was followed by two bands of opaque material; these were therefore separately collected as fractions 4a and 4b; a similar distribution had occasionally been seen in earlier experiments. Fraction 5 was the clear area at the bottom of the tube, as in the first experiment.

TABLE 1

5-HYDROXYTRYPTAMINE (5HT) AND ADENOSINETRIPHOSPHATE (ATP) IN FRACTIONS OF SMALL INTESTINE OF RESERPINE-TREATED DOGS AFTER DENSITY GRADIENT CENTRIFUGATION

Concentrations expressed as n moles/g tissue

Expt. no. 1

Fraction no.	5HT	ATP	Ratio: 5HT/ATP
1	0·001	None	—
2	0·069	1·0	0·069
3	0·026	None	—
4	0·026	<0·05	—
5	None	None	—

Expt. no. 2

Fraction no.	5HT	ATP	Ratio: 5HT/ATP
1	0·075	None	—
2	1·64	2·54	0·65
3	0·07	None	—
4a	0·30	None	—
4b	0·25	None	—
5	0·46	None	—

The results of these experiments are shown in Table 1. In both experiments the total amounts of 5-hydroxytryptamine recovered were low (<4%) in comparison with those previously observed in non-reserpinized dogs. The degree of depletion achieved in experiment 1 was more severe. In particular, fraction 4 (or fractions 4a and 4b), usually rich in amine (Prusoff, 1960), contained no or little amine; in both experiments the amounts of amine recovered in fraction 2 were higher. The content of adenosinetriphosphate of fraction 2 was somewhat low but within the range seen in non-reserpinized dogs, whereas the amounts of nucleotide present in the fraction normally rich in 5-hydroxytryptamine (fraction 4) were extremely low. This is shown in Table 2, in which the adenosinetriphosphate contents of fractions 2 and 4 are given for three normal (Prusoff, 1960) and two reserpine-treated animals. It may be mentioned here that fraction 4 had not disappeared under the influence of reserpine: macroscopically an opaque layer could be seen of an appearance quite similar to that in the animals not treated with reserpine.

TABLE 2
EFFECT OF RESERPINE, ADMINISTERED *IN VIVO*, ON THE ADENOSINETRIPHOSPHATE (ATP) CONTENT OF "MITOCHONDRIA" AND "5-HYDROXYTRYPTAMINE (5HT)-RICH GRANULES" FROM THE DOG SMALL INTESTINE

		ATP in n moles/g tissue		
Treatment	Expt. no.	Mitochondria (M)	5HT-rich granules (G)	Ratio M/G
None	1	3·96	1·98	2·0
None	2	5·52	1·73	3·2
None	3	0·93	0·97	1·0
Reserpine	1	1·01	<0·05	>20
	2	2·54	None	—

DISCUSSION

Earlier experiments (Prusoff, 1960) had shown that by density gradient centrifugation two distinct fractions could be obtained from the mucous membrane of the dog's small intestine that were rich in adenosinetriphosphate. Only one of these fractions was also rich in 5-hydroxytryptamine. In this fraction the molar ratio, amine : adenosinetriphosphate, was similar to that found in the adrenal medulla. It was suggested that most of the nucleotide in this fraction was connected with amine storage, whereas the nucleotide in the upper layer of the density gradient was not associated with 5-hydroxytryptamine.

The present experiments support this interpretation. After treatment with reserpine the layer normally rich in 5-hydroxytryptamine contained even less amine than the "mitochondrial" layer. Table 2 shows that the nucleotide believed to be associated with 5-hydroxytryptamine had disappeared, whereas the adenosinetriphosphate content of the "mitochondrial" fraction had remained at normal levels. This is in agreement with the idea that only part of the total nucleotide is directly concerned with amine storage.

Although 5-hydroxytryptamine and adenosinetriphosphate contents of the fraction normally rich in amine had disappeared after reserpine treatment, there was no concomitant change in the opaqueness of this fraction. There are several possible explanations. The release of amine and loss of adenosinetriphosphate may occur without disruption of the granule. This would imply that the density of these granules is not due to their amine and/or nucleotide content. Another possibility is that this fraction is still heterogeneous and that the amine-carrying granules represent only a fraction thereof. Electron-microscopic examination of this fraction did in fact reveal a lack of homogeneity (Meek & Barer, personal communication).

In the chromaffin tissue of the adrenal medulla, loss of catechol amines after reserpine is accompanied by a loss in adenosinetriphosphate (Schümann, 1958; Kirpekar, Goodlad & Lewis, 1958; Hillarp, 1960; Burack, Weiner & Hagen, 1960). Thus, in both the chromaffin and the "enterochromaffin" cell, reserpine reproduces a loss in nucleotide as well as a loss of amine. This raises the possibility that the disappearance of adenosinetriphosphate is the common cause of the releasing action of reserpine, an idea already discussed by Bein (1957, 1960). It must be remembered, however, that reserpine also leads to a release of amine from blood platelets, but there appears to be no concomitant disappearance of adenosinetriphosphate

(Born, Ingram & Stacey, 1958). Nevertheless, although there is good reason to believe that in the platelets the nucleotide is also involved in storage of amine, the low molar ratio, 5-hydroxytryptamine:adenosinetriphosphate, makes it likely that in the platelets there is a great excess of nucleotide that may not be involved in amine storage.

The many analogies between the chromaffin and the enterochromaffin cells are strengthened by the results that have been described here.

I am grateful to Dr H. Blaschko for the hospitality extended to me during my stay in Oxford. I also acknowledge help given by Mr H. W. Ling, Mrs Rosemary Bonney and Miss Helen Latham in these experiments.

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THE DISTRIBUTION OF SOME QUATERNARY AMMONIUM SALTS IN THE PERIPHERAL NERVOUS SYSTEM OF CATS IN RELATION TO THE ADRENERGIC BLOCKING ACTION OF BRETYLIUM

BY

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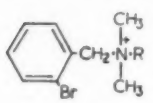
The concentrations of bretylium in the peripheral nervous system of cats after subcutaneous dosage have been compared with those drugs bearing either chemical or pharmacological resemblance to bretylium. *o*-Bromobenzyltrimethylammonium iodide (383C57), a chemical homologue of bretylium with feeble adrenergic blocking activity, selectively accumulated in adrenergic neurones to attain concentrations comparable with those found for bretylium, but it did not persist in the neurones. {2-(4-Benzoyl-2,6-dimethylphenoxy)ethyl}trimethylammonium, the 4-benzoyl derivative of 2,6-xylyl choline ether (172C58), a pharmacological analogue of bretylium but with little chemical resemblance, had a less marked selective affinity for adrenergic neurones and the concentrations after an effective dose were much lower than found for bretylium. Pentacynium {N^1-5-cyano-5,5-diphenylpentyl)-$N^1N^1N^2$-trimethylethylene-1-ammonium-2-morpholinium di-iodide}, a bis-quaternary ammonium-type ganglion blocking agent, had no selective affinity for adrenergic neurones. Bretylium was not displaced from adrenergic neurones by a subsequent dose of 172C58. The potency and duration of local anaesthetic action of bretylium, 383C57 and 172C58 were roughly parallel to the potency and duration of their adrenergic blocking action. The results are discussed in relation to the role of selective accumulation in adrenergic neurones by bretylium in its adrenergic blocking activity.

Selective inhibition of the peripheral adrenergic system by bretylium (I ; R = ethyl) (Boura & Green, 1959) seems to be related to a specific affinity of the drug for adrenergic neurones (Boura, Copp, Duncombe, Green & McCoubrey, 1960b). The rise and fall of concentration of the ^{14}C-labelled drug in the adrenergic nerves and ganglia of cats showed a fair parallelism to the time course of the adrenergic block, and high concentrations of the drug persisted in these structures when levels in other organs had fallen considerably.



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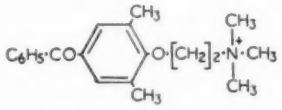

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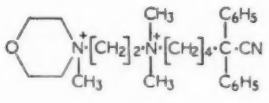

III

{2-(4-Benzoyl-2,6-dimethylphenoxy)ethyl}trimethylammonium, the 4-benzoyl derivative of 2,6-xylyl choline ether, referred to as 172C58 (II), has a similar pharmacological effect to bretylium in cats at one-tenth of the subcutaneous dosage

needed for bretylium. The effects are more rapid in onset. The adrenergic neurone block caused by 172C58, in the *in vitro* preparation described by Finkleman (1930), unlike that caused by bretylium, is readily abolished by washing. The drug has little adrenergic blocking action in man (Boura, Coker, Copp, Duncombe, Elphick, Green & McCoubrey, 1960a). By contrast, *o*-bromobenzyltrimethylammonium, referred to as 383C57 (I; R = methyl), has very little adrenergic neurone blocking action in cats in spite of its close chemical resemblance to bretylium (Boura & Green, unpublished). The distribution of these quaternary salts in cats has therefore been compared to those of bretylium and pentacynium (III), a ganglion blocking agent of the bis-quaternary ammonium type (Adamson, Billinghurst, Green & Locket, 1956).

METHODS

Chemicals

The ^{14}C-labelled drugs were prepared by Dr F. C. Copp (383C57 and 172C58) and Mr J. W. Billinghurst (pentacynium) by quaternizing the appropriate tertiary amine with [^{14}C]-methyl iodide. Pentacynium was labelled at the morpholine nitrogen atom. They were checked for purity as described for bretylium (Boura *et al.*, 1960b).

o-Bromobenzyltrimethylammonium (383C57). The product had a specific activity of 1 mC/mmole. For doses of 10 mg/kg it was diluted with an equal part of inactive carrier. For the 50 mg/kg dose it was diluted with ten parts of carrier.

{2-(4-Benzoyl-2,6-dimethylphenoxy)ethyl}trimethylammonium (172C58). The product, 5 mC/mmole, was used without dilution by carrier.

Pentacynium. The product, 1 mC/mmole, was used without dilution by carrier.

Experimental

Cats received the drugs subcutaneously and were killed at the stated times by exsanguination from the aorta under ether anaesthesia. Doses were, for 383C57, 10 and 50 mg/kg; for 172C58, 1 mg/kg; and for pentacynium, 2 mg/kg. Tissue sampling and radioactivity determinations were performed as described for bretylium (Boura *et al.*, 1960b).

Cats under chloralose anaesthesia were prepared for preganglionic stimulation of the superior cervical sympathetic nerve on both sides (10 cycles/sec for 1 min every 3 min), and contractions of the nictitating membranes were recorded on a smoked drum. The response was constant at the fourth stimulation. Labelled bretylium (2.5 mg/kg) was given intravenously and after 5 min a further 2.5 mg/kg. When the height of the trace on the drum was reduced by half (in 15 to 20 min), the left superior cervical sympathetic nerve and ganglion and the vagus and nodose ganglia were excised and prepared for counting. A further 2.5 mg/kg bretylium was given, followed by either saline or 172C58 (unlabelled) (2 mg/kg). After 172C58 the membrane response was completely inhibited in a few minutes. With bretylium alone, inhibition was complete in a further 30 min. The right superior cervical sympathetic nerve and ganglia and the vagus and nodose ganglia were removed 50 min after the first dose and prepared for counting.

Local anaesthetic activity after intradermal injection into guinea-pigs was tested by the method of Bülbring & Wajda (1945).

RESULTS

Distribution of {2-(4-benzoyl-2,6-dimethylphenoxy)ethyl}trimethylammonium (172C58). The general pattern of distribution of 172C58 in the glands and larger organs of cats killed at 2, 12 and 18 hr after 1 mg/kg is shown in Table 1. The dose fully relaxed the nictitating membranes of all the four cats, within 30 min of

TABLE 1

DISTRIBUTION OF {2-(4-BENZOYL-2,6-DIMETHYLPHENOXY)ETHYL}TRIMETHYL-AMMONIUM IODIDE IN CAT TISSUES

1 mg/kg of the *N*-[^{14}C]-methyl-labelled drug was given subcutaneously. Concentrations are expressed in mμmoles/g wet tissue. 1 mμmole=0·439 μg 172C58

	Time in hr			
Tissue	2	12	18	18
Adrenal gland	0·3	0·4	0·0	0·2
Parotid gland	4·2	1·3	0·5	0·2
Thyroid gland	1·7	—	0·1	—
Heart	6·4	3·4	0·8	0·8
Kidney	2·2	1·6	0·8	—
Spleen	2·3	1·8	0·9	0·4
Liver	9·5	6·0	4·3	4·4
Lung	0·9	1·5	1·3	1·2
Plasma	0·1	0·06	0·4	—

injection. The concentrations of 172C58 in a variety of other tissues, not given in Table 1, at 18 hr after the dose were between 0.1 and 0.4 mμmoles/g. There was none in perirenal fat.

The drug appeared to be rapidly absorbed by the liver, which achieved the highest concentration of any tissue examined, and relatively high levels tended to persist. A relatively high proportion of the dose was found in the bile of one cat killed at 18 hr (130 μg.≡6% of the dose). Unfortunately, urine was not obtainable from this animal. The kidney contained a relatively small proportion of the dose at all time intervals studied. In the one specimen of urine obtained at 18 hr there was 350 μg≡20% of the dose.

TABLE 2

DISTRIBUTION OF {2-(4-BENZOYL-2,6-DIMETHYLPHENOXY)ETHYL}TRIMETHYL-AMMONIUM IODIDE IN NERVES AND GANGLIA OF CATS

1 mg/kg of the *N*-[^{14}C]-methyl-labelled drug given subcutaneously. Concentrations are expressed in mμmoles/g wet tissue. 1 mμmole=0·439 μg 172C58

	Time in hr			
Tissue	2	12	18	18
Sympathetic ganglia				
Superior cervical	3·7	5·4	5·1	4·6
Stellate	4·7	3·8	2·0	2·8
Coeliac	5·6	4·9	2·5	3·0
Superior mesenteric	5·4	6·7	1·2	—
Inferior mesenteric	4·0	3·4	1·9	—
Other ganglia				
Sympathetic chain	1·0	1·0	1·8	—
Dorsal root	0·8	0·9	0·6	—
Nodose	1·0	1·3	0·3	1·0
Adrenergic nerves				
Post-ganglionic cervical	0·9	2·0	2·0	2·2
Inferior cardiac	1·7	1·3	2·3	2·1
Splenic and gastric	0·4	1·4	0·8	—
Cholinergic nerves				
Pre-ganglionic cervical	0·4	0·4	0·2	—
Splanchnic	0·2	0·7	0·0	—
Optic	—	—	0·2	—
Vagus	0·1	—	0·2	—
Phrenic	—	—	0·1	—
Sciatic	—	0·1	0·04	—

The concentrations of 172C58 found in the sympathetic ganglia were higher than in most tissues (1.2 to 5.6 mμmoles/g) and the drug tended to persist in these structures (Table 2). By contrast, the adrenal glands took up very little of the drug. Concentrations in the adrenergic nerves were not outstandingly high, though they exceeded those in non-adrenergic nerves. Thus the concentrations in the pre-ganglionic cervical sympathetic nerves were one-fifth to one-tenth of those in the post-ganglionic nerves at 12 and 18 hr after the dose. Ganglia other than those of the sympathetic system contained up to 1.3 mμmoles/g, the highest being in the nodose ganglion at 12 hr after the dose. Among tissues not mentioned in the table, the ciliary and gasserian ganglia, the cerebral and cerebellar cortices and the spinal cord did not accumulate the drug. The hypothalamus had a trace (0.1 mμmole/g) at 12 hr. There was no evidence for radioactive metabolic products in urine after autoradiography of chromatograms of whole urine or trichloroacetic acid extracts of liver in *s*-butanol–acetic acid–water mixture (12:5:3).

Distribution of o*-bromobenzyltrimethylammonium (383C57).* The concentration of 383C57 in representative tissues at 3, 12 and 18 hr after 10 mg/kg is summarized in Tables 3 and 4. One of the three cats used, that killed at 12 hr, showed just noticeable relaxation of the nictitating membranes. No relaxation had been seen in a previous series of five cats that had received the same dose level of unlabelled drug. After 50 mg/kg a near maximal relaxation of the nictitating membranes occurred, but they had almost regained their normal tone when the animal was killed at 12 hr after the dose. A similar brief effect was seen in three other cats receiving the unlabelled drug at the same dose level. This near toxic dose caused salivation and vomiting.

Concentrations of 383C57 in the larger organs after 10 mg/kg were highest at 3 hr after the dose, though the blood levels were fairly constant in all three cats used. Fairly high concentrations were found in the adrenal glands and (in one cat only) in the thyroid.

TABLE 3

DISTRIBUTION OF *o*-BROMOBENZYLTRIMETHYLAMMONIUM IODIDE (383C57) IN CAT TISSUES

10 mg/kg of the *N*-[^{14}C]-methyl-labelled drug was given subcutaneously. Concentrations are expressed in mμmoles/g wet tissue. 1 mμmole=0·356 μg 383C57

Tissue	Time in hr		
	3	12	18
Adrenal gland	67	59	39
Parotid gland	—	0	6
Thyroid gland	—	120	2
Heart	63	13	9
Kidney	21	9	5
Spleen	44	17	16
Liver	51	9	6
Lung	—	10	6
Blood	7	8	9
Skeletal muscle	18	12	12
Area postrema	7	5	12
Cerebral cortex	1	0	—
Cerebellar cortex	0	0	—
Hypothalamus	0	7	0
Spinal cord	—	0	0

TABLE 4

DISTRIBUTION OF *o*-BROMOBENZYLTRIMETHYLAMMONIUM IODIDE (383C57) IN NERVES AND GANGLIA OF CATS

10 mg/kg of the *N*-[^{14}C]-methyl-labelled drug was given subcutaneously. Concentrations are expressed in mμmoles/g wet tissue. 1 mμmole=0·356 μg 383C57

Tissue	Time in hr			50 mg
	3	12	18	12
Sympathetic ganglia				
Superior cervical	149	164	105	312
Stellate	213	468	97	219
Inferior mesenteric	—	39	47	93
Coeliac	213	120	72	207
Other ganglia				
Dorsal root	21	6	5	15
Nodose	17	9	39	18
Ciliary	63	—	2	25
Adrenergic nerves				
Post-ganglionic cervical	28	76	63	103
Inferior cardiac	193	176	71	162
Splenic and gastric	—	—	70	193
Hypogastric	71	0	30	203
Cholinergic nerves				
Pre-ganglionic cervical	18	14	20	49
Splanchnic	—	14	—	—
Vagus	13	9	23	12
Phrenic	—	13	3	22
Sciatic	—	2	7	9

The highest concentrations were found in the sympathetic ganglia at 12 hr after the dose. The levels in the cat killed at 18 hr were much lower. Other ganglia had lower concentrations of the drug. Similarly, the levels in adrenergic nerves considerably exceeded those in non-adrenergic nerves.

There was good recovery of the dose from urine in the cat killed at 18 hr (76%). Chromatography of this urine and of a trichloroacetic acid extract of the liver in *s*-butanol–acetic acid–water mixture (12:5:3) followed by autoradiography revealed only one radioactive spot at R_F 0.8 corresponding to the unchanged drug.

TABLE 5

DISTRIBUTION OF PENTACYNIUM IN THE NERVES AND GANGLIA OF CATS

2 mg/kg of *N*-[^{14}C]-methyl-labelled pentacynium given subcutaneously. Tissues were sampled at 3 hr after the dose. Concentrations in mμmoles/g wet tissue. 1 mμmole=0·679 μg pentacynium iodide

Tissue	Concentration
Ganglia	
Superior cervical	1·4
Stellate	1·9
Superior mesenteric	0·0
Coeliac	Trace
Nodose	4·5
Ciliary	5·5
Nerves	
Post-ganglionic cervical sympathetic	Trace
Splenic and gastric	2·0
Hypogastric	3·8
Inferior cardiac	Trace
Greater splanchnic	3·8
Vagus	1·3
Pre-ganglionic cervical sympathetic	5·1
Sciatic	Trace

The level of 383C57 in nerves and ganglia after 50 mg/kg was roughly doubled compared to the 10 mg/kg dose at the same time interval. Notable exceptions were the stellate ganglion with a lower concentration and the hypogastric nerve with a much higher concentration.

Distribution of pentacynium. The dose of pentacynium (2 mg/kg) had relaxed the nictitating membranes and dilated the pupils maximally when the cat was killed at 3 hr after the dose. The levels in nerves and ganglia are given in Table 5. Counting rates were low and errors in estimation of the concentrations amounted to ±10% for the more active samples to ±50% for those of low activity. There was little radioactivity in the larger organs, except kidney. The result indicates that pentacynium had no affinity for the adrenergic ganglia or their post-ganglionic nerves in excess of that shown for cholinergic nervous tissue.

Local anaesthetic activity. The duration of local anaesthesia after intradermal injection of bretylium, 172C58 and 383C57 is shown in Fig. 1. While 383C57 was equiactive to bretylium in potency as measured by maximum effect, it had little

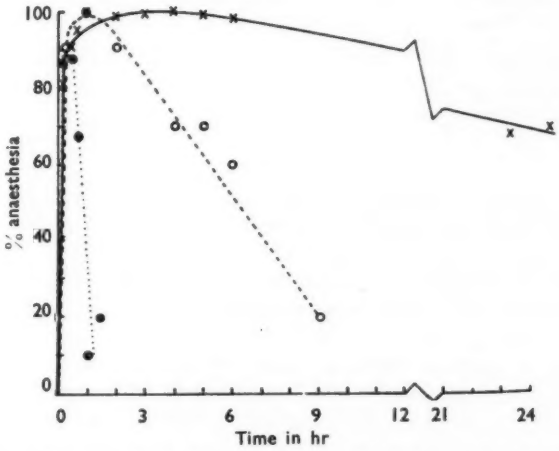

Fig. 1. Local anaesthetic activity of 3% *o*-bromobenzyltrimethylammonium (383C57) (●.... ●), 0·3% {2-(4-benzoyl-2,6-dimethylphenoxy)ethyl}trimethylammonium (172C58) (○ – – – ○), and 3% bretylium (X——X) after intradermal injection in guinea-pigs.

persistence. Indeed, the persistence of the local anaesthetic effect of bretylium is outstanding. Dose for dose, 172C58 had greater potency than bretylium, but at an equipotent dose its persistence was much less marked.

Influence of {2 - (4 - benzoyl - 2,6 - dimethylphenoxy)ethyl} trimethylammonium (172C58) on the accumulation of bretylium. Table 6 shows that the concentrations of bretylium in the superior cervical ganglion and its post-ganglionic nerve at 50% inhibition of contraction of the nictitating membranes were 70 mμmoles/g (mean of 4 results) and 18 mμmoles/g (mean of 2 results) respectively. At full inhibition of contraction a mean concentration of 148 mμmoles/g was found in the ganglion,

TABLE 6

CONCENTRATION OF BRETYLIUM IN NERVES AND GANGLIA OF CATS AT PARTIAL AND FULL INHIBITION OF THE CERVICAL SYMPATHETIC NERVE AND THE LACK OF INFLUENCE OF {2-(4-BENZOYL-2,6-DIMETHYLPHENOXY)ETHYL}TRIMETHYLAMMONIUM (172C58)

Cats under chloralose anaesthesia given 2 × 2·5 mg/kg doses of [^{14}C]-labelled bretylium intravenously. The left cervical sympathetic and vagus nerves were sampled at approx. 50% inhibition. A further 2·5 mg/kg labelled bretylium was given, followed by either saline or 172C58 (2 mg/kg) intravenously and the nerves of the right side sampled at 50 min. Concentration in mμmoles/g

	Bretylium+saline		Bretylium+172C58	
Tissue	At 50% block	At full block	At 50% block	At full block
Superior cervical ganglion	79, 15	125, 171	101, 86	204, 139
Post-ganglionic cervical sympathetic nerve	19, —	17, —	79, —	39, —
Pre-ganglionic cervical sympathetic nerve	—, 7	—, 22	—, 13	—, 18
Nodose ganglion	20, 32	28, 39	33, 29	38, 28
Vagus	8, 8	9, 22	8, 11	12, 13
Stellate ganglion	— —	153, —	— —	212, —
Coeliac ganglion	— —	154, —	— —	237, —
Blood	4, —	7, 2·4	20, —	4, 4

and this was not significantly altered (172 mμmoles/g) by a dose of 172C58. A maximum concentration in the nodose ganglia and the vagus nerves (about 10 mμmoles/g) was achieved virtually within 20 min.

DISCUSSION

The marked affinity of bretylium for the peripheral adrenergic neurones of cats (Boura *et al.*, 1960b) undoubtedly contributes to its adrenergic neurone blocking action in this species. This discussion is concerned mainly with the problem whether the mechanism of this selective localization is an integral part of the adrenergic blocking mechanism or whether the localization is a fortuitous property ensuring the presence of adequate levels of drug at its site of action.

Pentacynium, a ganglion blocking agent, inhibits the effect of pre-ganglionic stimulation of the superior cervical sympathetic nerve, but its mode of action clearly differs from that of bretylium (Boura & Green, 1959). Its failure to accumulate selectively in adrenergic ganglia suggests that the localization of bretylium is a

TABLE 7

COMPARATIVE ACTIVITIES OF {2-(4-BENZOYL-2,6-DIMETHYLPHENOXY)ETHYL}-TRIMETHYLAMMONIUM (172C58), BRETYLIUM AND *o*-BROMOBENZYLTRIMETHYLAMMONIUM (383C57)

	172C58	Bretylium	383C57
Adrenergic neurone			
Accumulation in	Low	High	High
Persistence in	Moderate	High	Low
Persistence of block	Moderate	High	Low
Finkleman preparation			
Activity	High	Moderate	Low
Persistence after washing	Low	High	Low
Local anaesthesia			
Activity	High	Moderate	Low
Persistence	Moderate	High	Low

specific property of this type of adrenergic blocking drug. The results with *o*-bromobenzyltrimethylammonium and {2-(4-benzoyl-2,6-dimethylphenoxy)ethyl}-trimethylammonium, however, show that any relation between adrenergic blocking mechanism and selective localization is not a simple one.

In Table 7, ratings have been given to the degrees of selective accumulation in adrenergic nervous tissue relative to dosage for *o*-bromobenzyltrimethylammonium, {2-(4-benzoyl-2,6-dimethylphenoxy)ethyl}trimethylammonium and bretylium, to their relative potencies and persistence of adrenergic block in the whole animal and to their intrinsic adrenergic blocking activity as indicated by the Finkleman preparation. {2-(4-Benzoyl-2,6-dimethylphenoxy)ethyl}trimethylammonium appears to have an intrinsic activity some ten times that of bretylium and about fifty times that of *o*-bromobenzyltrimethylammonium. The levels of {2-(4-benzoyl-2,6-dimethylphenoxy)ethyl}trimethylammonium in adrenergic neurones were about one-hundredth of those found for bretylium at comparable pharmacological effect, but were only about five times those found in cholinergic nerves and ganglia. There is nevertheless a clear selective localization of {2-(4-benzoyl-2,6-dimethylphenoxy)-ethyl}trimethylammonium in adrenergic nervous tissue. Conversely, *o*-bromobenzyl-trimethylammonium, with very low intrinsic activity, accumulated in adrenergic neurones to about the same degree as bretylium, but it lacked persistence. Since all three drugs accumulated in tissues to levels considerably higher than those in blood, there must be a degree of non-specific accumulation in adrenergic neurones analogous to that in liver or cholinergic nerves. Table 8 shows the excess of

TABLE 8

"SPECIFIC" ACCUMULATION OF QUATERNARY AMMONIUM SALTS IN THE SUPERIOR CERVICAL GANGLIA AND POST-GANGLIONIC CERVICAL SYMPATHETIC NERVES

The figures are the excess concentration {2-(4-benzoyl-2,6-dimethylphenoxy)ethyl}trimethylammonium (172C58), *o*-bromobenzyltrimethylammonium (383C57) and bretylium in mμmoles/g wet tissue for (a) superior cervical ganglia over nodose ganglia, (b) post- over pre-ganglionic cervical sympathetic nerve

	172C58			383C57			Bretylium		
Time in hr	2	12	18	3	12	18	3	12	18
Superior cervical ganglia	2·7	4·1	4·8	132	155	166	159	255	860
Post-ganglionic cervical sympathetic nerve	0·5	1·7	1·8	10	52	43	14	49	620

concentration in the superior cervical ganglia over that in the nodose ganglia and the excess in post-ganglionic cervical sympathetic nerves above that in the pre-ganglionic nerves at the time intervals studied. The figures indicate that *o*-bromobenzyltrimethylammonium was little inferior to bretylium in this "specific" accumulatory capacity up to 12 hr after the dose, but that it did not continue to accumulate after this time as did bretylium. "Specific" accumulation of {2-(4-benzoyl-2,6-dimethylphenoxy)ethyl}trimethylammonium apparently occurred rapidly to a low constant level. There is clearly a dissociation between intrinsic activity and degree of specific accumulation.

The relatively low intrinsic activity of bretylium compared with {2-(4-benzoyl-2,6-dimethylphenoxy)ethyl}trimethylammonium is probably compensated by its greater

persistence, while *o*-bromobenzyltrimethylammonium is probably virtually inactive because its very low intrinsic activity is not compensated by persistence. The mechanism of persistence is conceivably distinct from that of *initial* accumulation, which indeed may be no more specific than occurs in other organs. On these grounds the accumulation of bretylium could be the consequence rather than the cause of adrenergic block, and its binding in the tissue could result from some change in the tissue. The accumulation of bretylium was not influenced by a subsequent dose of {2 - (4 - benzoyl - 2,6 - dimethylphenoxy)ethyl}trimethylammonium—it was neither displaced nor was accumulation enhanced. Though application of bretylium to sensory nerve endings in the skin causes persistent local anaesthesia, there is clearly no marked accumulation of bretylium by these nerve endings after systemic administration, since bretylium does not cause generalized cutaneous sensory loss. It seems reasonable to conclude that persistence, which can lead to retention and slow accumulation, is more related to adrenergic blocking mechanism than accumulation itself and that this persistence is likely to be a reflection of specific binding at some hypothetical receptor. Thus, {2-(4-benzoyl-2,6-dimethylphenoxy)-ethyl}trimethylammonium, with a high intrinsic activity, has a relative inability to persist, for it can be readily washed out of *in vitro* preparations, and this may go some way to explain its failure to effect adrenergic block in man.

We are indebted to Dr A. C. White and Dr H. T. Openshaw for their encouragement in these investigations, and to Messrs P. J. Moore and A. E. Lakeman for technical assistance.

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RATE OF ANTAGONISM OF TUBOCURARINE BY POTASSIUM IONS

BY

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The rate at which potassium ions antagonized the neuromuscular block produced by tubocurarine has been examined in isolated rat diaphragm muscle preparations. The half-time was dependent on the thickness of the muscle. In thick muscles (550 to 650 μ) the rate of action could be largely accounted for by the time which the potassium took to diffuse through the interstitial fluid to produce an increase in concentration in the immediate environment of the muscle fibre.

It is well known that addition of potassium antagonizes the blocking action of tubocurarine in skeletal muscle (Wilson & Wright, 1936; Quilliam & Taylor, 1947). When this is studied in isolated muscle, the process takes a measurable time for completion, and it was of interest to determine how far diffusion of potassium through the interstitial spaces to its site of action could account for the time relations.

METHODS

The solution bathing the diaphragm was similar to that used by Creese (1954) except that the potassium content was reduced to 1.5 mM. The fluid was bubbled with a mixture of 5% carbon dioxide and 95% oxygen, and the temperature of the bath was maintained at 38° C.

The left hemi-diaphragm from an albino rat weighing 100 to 150 g was removed, attached to a holder and immersed in the solution in such a manner that single maximal shocks could be delivered alternately to the nerve and muscle, using the procedure described by Holmes, Jenden & Taylor (1951) except that condenser shocks of 0.02 μF were used for the nerve and 2 μF for the muscle. The responses were recorded on smoked paper using a spring-loaded lever. Stimulation was continued throughout the experiments, and the magnitude of the responses obtained by direct and indirect stimulation was very similar. As direct stimulation was almost unaffected by tubocurarine, an estimate of the degree of block could be made at any time during the experiment.

The muscle was later removed from the bath, outlined on squared paper and weighed. The thickness was calculated, taking the specific gravity as 1.055 (Creese, 1954) and expressing the result to the nearest 5 μ.

RESULTS

The height of contraction of the muscle after immersion in the solution containing 1.5 mM potassium showed an initial decline. The solution was changed repeatedly until the height of contraction became steady, usually after 2 hr.

Sufficient tubocurarine was then added to produce a steady partial neuromuscular block. In the experiment illustrated in Fig. 1, 20 μg of tubocurarine had been added to the bath containing 40 ml. solution. The contractions which followed stimulation of the nerve became steady 2 hr after addition of the tubocurarine at a contraction height of 5% of the control obtained by direct stimulation of the muscle. At zero time, 1 ml. of a solution containing potassium chloride was added beneath the meniscus, so that the potassium content of the bath was suddenly raised from 1.5 to 7.4 mM. The solution which was added was obtained by dissolving potassium

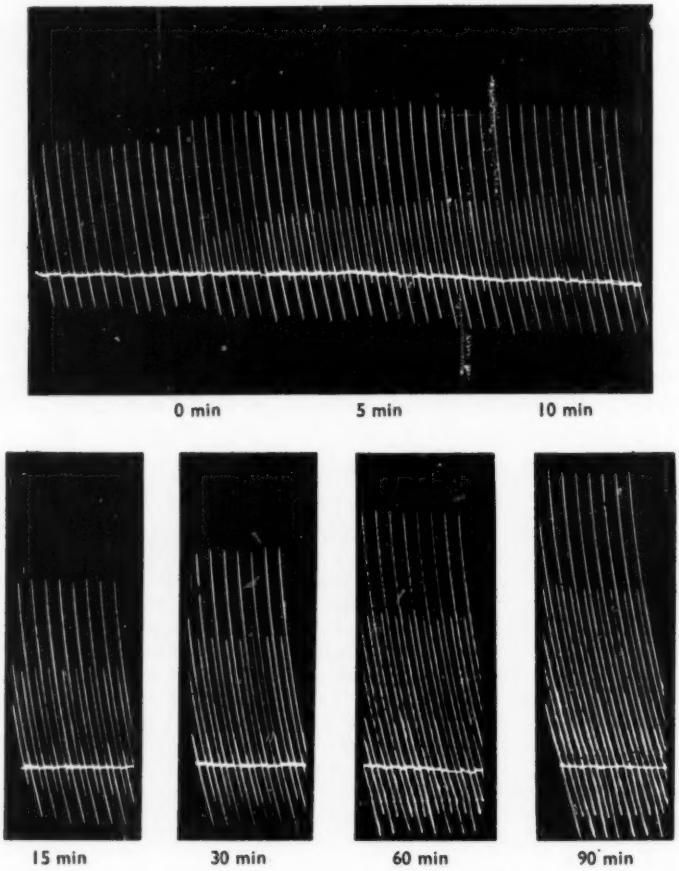

Fig. 1. Effect of potassium on the neuromuscular block produced by tubocurarine (0·5 μg/ml. administered 2 hr previously) in rat diaphragm. At zero time, the potassium concentration in the bath was increased from 1·5 to 7·4 mM. Bath volume, 40 ml. Temperature, 38° C. In this record stimulation was alternated to the muscle directly and to the phrenic nerve. The indirect responses, partially blocked by tubocurarine, are smaller than those in response to direct muscle stimulation.

chloride in some of the original solution which had been prepared ; it also contained tubocurarine (0.5 μg/ml.) and was warmed to 38° C before being added to the bath.

After addition of the potassium, the height of contraction produced by direct stimulation was slightly increased. There then followed a slow rise in the response to direct stimulation, taking about 1.5 hr to reach a maximum. The response to indirect stimulation also increased, rapidly during the first few min but slower later, reaching a maximum in 1.5 hr.

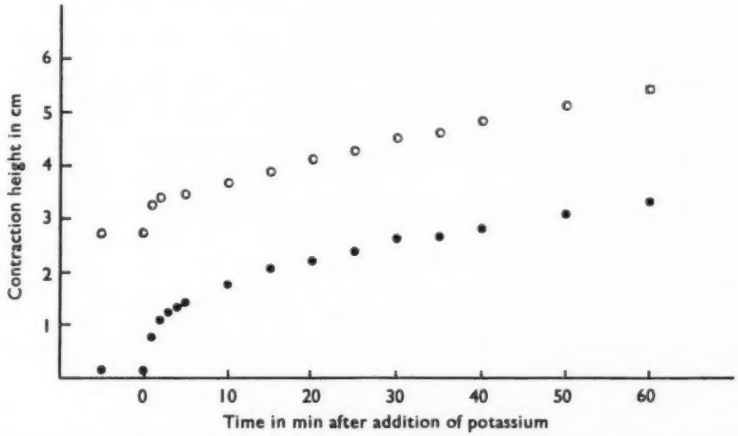

Fig. 2. Contraction heights plotted from tracing of Fig. 1. ○, Direct stimulation; ●, nerve stimulation. The abscissa gives the time after the increase of potassium concentration in the bath from 1·5 to 7·4 mM.

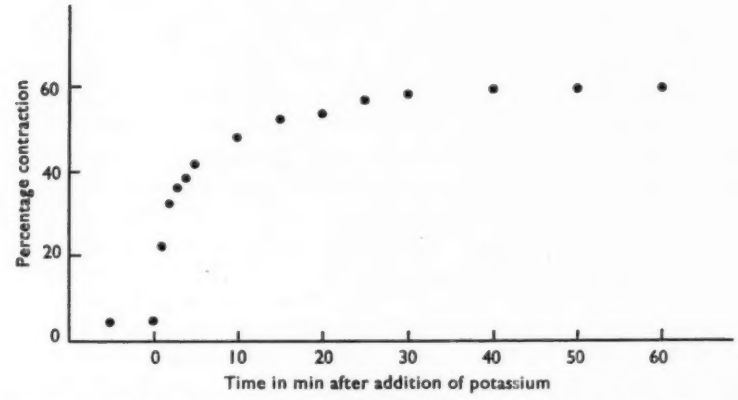

Fig. 3. Contraction height due to nerve stimulation expressed as a percentage of response obtained by direct stimulation of the muscle, from Figs. 1 and 2. For further explanation, see text. Abscissa gives time after the increase of potassium concentration in the bath from 1·5 to 7·4 mM.

In Fig. 2 the heights of contraction have been plotted during the first 60 min. If the height of the indirect response (Fig. 2, solid circles) is expressed as a percentage of the height of the direct muscle response (Fig. 2, open circles) at the time under consideration, a value for percentage contraction is obtained. Fig. 3 relates percentage contraction in response to nerve stimulation to time in the experiment illustrated in Fig. 1, and shows the rapid initial increase in contraction, followed by a slower

TABLE 1

OBSERVED HALF-TIMES SEEN WITH 7·4 mM POTASSIUM IN THE BLOCK BY TUBOCURARINE IN RAT DIAPHRAGM MUSCLE

Estimate of muscle thickness (μ)	Observed half-time (sec)
460	33
460	40
490	28
500	44
540	118
580	78
600	136
600	122
625	163
640	148
640	165
650	150

rise to maximum effect after the potassium concentration of the fluid bathing the preparation was suddenly raised to 7.4 mM at zero time.

In Fig. 3 the contraction following nerve stimulation increased from 5% at 0 min to a maximum of 59% of the control after 40 min. Half the maximum recovery of twitch height was 32% ([5+59]/2) and was achieved in 118 sec. Table 1 gives

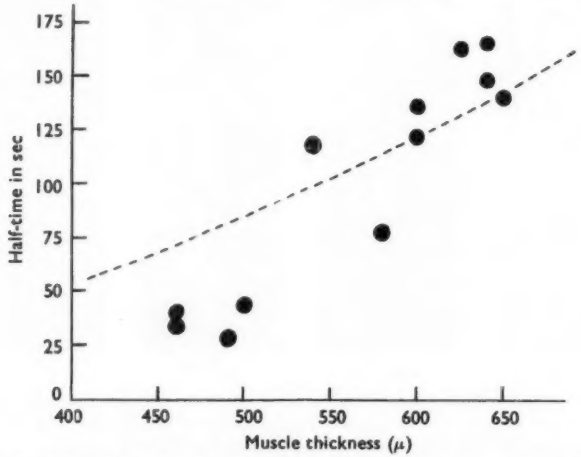

Fig. 4. The relation between calculated muscle thickness and the half-time required for the action of potassium on tubocurarine. The dotted line gives the time for the increase of potassium in the interspaces to reach half its final value, calculated on the basis described in the text.

the time of half-recovery in 12 experiments and the estimated thickness of the preparations. In Fig. 4 the results have been plotted to show the relation between the thickness of the muscle and the half-time for the action of potassium. It was found that the thickness of the muscle had a very marked effect on the time taken for potassium to antagonize tubocurarine.

DISCUSSION

The ratio of the height of contraction to indirect stimulation to that following direct stimulation was used as an indication of the fraction of fibres responding and hence the extent of the block. The increase of height of contraction to direct stimulation was assumed to be due to an increase in the force of contraction of each of the muscle fibres, rather than to an increase in the number of fibres responding because the directly applied shock was maximal. It was also assumed that the response plotted in Fig. 3 would be half-way to its final value when half the extra potassium had entered the tissue interspaces.

The time required for half the extra potassium to accumulate in the interstitial spaces may be calculated. It follows from Hill (1928) that for diffusion in the interspaces of a flat muscle Dt/d^2 is 0.196, where t is the half-time, d is the diffusion path and is half the thickness, and D is the relevant diffusion coefficient of potassium. Creese (1954) measured D', the apparent diffusion coefficient of potassium in diaphragm, and obtained the value of 5.2×10^{-6} cm^2 sec^{-1} by following the movements of ^{42}[K] into a solution containing high potassium (118 mM). In the experiment shown in Fig 1, some potassium would enter the fibres as well as the interspaces. It has been shown by Csapo & Wilkie (1956) that, under such conditions, the effective diffusion coefficient for potassium accumulation in the interspaces would be aD', where a is the fraction by volume of the interspaces. This fraction is 0.28 for diaphragm muscle (Creese, 1954). By applying these findings, theoretical half-times have been calculated for various thicknesses of the rat diaphragm muscle and are expressed by the dotted line in Fig. 4. In this procedure the muscle is treated as a slab of relatively large area. The experimental points fell near the calculated line with thick muscles (550 to 650 μ), but fell below with muscles thinner than 500 μ. Thus the unblocking action of potassium ions proceeds more rapidly in the case of thin muscles than can be accounted for by the simple theory.

Csapo & Wilkie (1956) studied the rate at which high concentrations of potassium ions produced reversible inexcitability in frog muscle, and concluded that interfibre diffusion was responsible. In their studies the concentration of potassium in the interspaces had to reach a critical value (9 mM) before a fibre was affected. In the experiment shown in Fig. 1, there was a critical concentration of tubocurarine present, so that any increase in the potassium would diminish the neuromuscular block, and hence the kinetics would be different. In other respects, however, the same assumptions have been made, and in the calculations it has been considered that the interaction between tubocurarine and potassium is rapid for any one fibre. If potassium acts by depolarization, then this assumption is an approximation, for Hodgkin & Horowicz (1959) have shown that an increase of potassium ion concentration with constant chloride (which resembles the experiment of Fig. 1) produces

immediate depolarization in the case of a single frog muscle fibre followed by a further slow and small depolarization so that the membrane potential eventually reaches the equilibrium value. This may explain the long time needed to produce a new steady neuromuscular block (Fig. 3). It would also be expected that the measured half-time would be somewhat greater than the calculated values, and the results of Fig. 4 for thick muscles (over 600 μ) are consistent with this.

In the case of thin muscle (below 500 μ) the potassium acted more rapidly than the simple theory predicted. Treatment in terms of a slab appeared to be no longer valid in the case of these small muscles with a relatively large surface-mass ratio. It was not possible to extend these studies over the range used by Conway & Fitzgerald (1942), who used diaphragm muscles up to 1 mm thick, because of the probability of anoxia occurring in the depths of the tissue (Creese, Scholes & Whalen, 1958) and consequent depolarization and potassium changes.

The potassium ions may exert their anti-curare action by depolarization, but other interactions or displacements of tubocurarine by potassium ions are not excluded by these experiments. Liley (1956) has found evidence that the liberation of transmitter may be increased by potassium. There is a resemblance between the factors governing the rate of action of tubocurarine and those governing the rate of action of potassium ions. It has already been pointed out (Holmes, Jenden & Taylor, 1951) that the site of action of tubocurarine is superficial; it probably does not penetrate any membranes which require an activation energy for the process, and the rate of action of tubocurarine can be accounted for largely by the time the drug takes to diffuse into the interfibre spaces (Creese, Taylor & Tilton, 1959).

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ANALGESIC-ANTIPYRETIC DRUGS AS INHIBITORS OF KALLIKREIN

BY

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The production of kallidin from guinea-pig and ox serum kallidinogen by the action of guinea-pig serum-kallikrein or human salivary kallikrein was inhibited by various analgesic-antipyretic drugs. This effect was obtained *in vitro* with concentrations of inhibitors which do not inhibit the smooth-muscle-stimulating action of the various polypeptides, but similar to the concentrations needed *in vivo* to obtain an anti-inflammatory action. The vasodepressor action of intravenously administered human salivary kallikrein in the anaesthetized dog was very markedly inhibited by the intravenous administration of doses of various analgesic-antipyretic drugs which only partially antagonized the responses to kallidin and bradykinin and which left the vasodepressor responses to histamine, acetylcholine and 5-hydroxytryptamine unaffected. In rabbits the accumulation of protein-bound dye at the site of intradermal injection of human salivary kallikrein and guinea-pig serum-kallikrein, but not of bradykinin and kallidin, was inhibited very markedly by the systemic administration of various analgesic-antipyretic drugs.

Analgesic-antipyretic drugs have been shown by Collier & Shorley (1960) to antagonize the bronchoconstrictor action of bradykinin in the anaesthetized guinea-pig, and Lecomte (1960) has demonstrated that in the rabbit phenylbutazone will prevent the fall in blood pressure and the local increase in capillary permeability in response to bradykinin.

It is widely recognized that analgesic-antipyretic drugs possess anti-inflammatory properties, both in rheumatoid diseases of man and also in experimentally induced inflammatory reactions in other species, but their mechanism of action is not clear (Smith, 1953). Bradykinin has been proposed by Hilton & Lewis (1955) as a possible mediator in certain inflammatory reactions, and their hypothesis is further substantiated by the fact that a chemically heterogeneous group of substances share the ability to inhibit both bradykinin and inflammatory reactions.

Several enzyme systems which are present in the mammalian body are reported to produce polypeptides either very closely related to, or identical with, bradykinin (Lewis, 1960a). If the liberation of bradykinin is an important mechanism in the production of an inflammatory reaction, then it seems likely that inhibitors of the enzymes which liberate bradykinin *in vivo* should act as anti-inflammatory agents. The experiments described in this paper are intended to test this possibility.

METHODS

Smooth-muscle stimulation. Isolated segments of the terminal ileum of guinea-pigs were suspended in a 20 ml. bath of oxygenated Tyrode solution at 33 to 34° C, and contractions recorded isotonically.

Vasodepressor activity. Dogs were anaesthetized with chloralose and the blood pressure recorded from the central end of the carotid artery by means of a cannula connected to a mercury manometer with a floating writing point. Drugs were administered by the femoral vein.

Accumulation of protein-bound dye. Bradykinin, kallidin or kallikrein was injected intradermally in the depilated flank skin of rabbits which had previously been injected with Evans blue dye intravenously, as described by Bhoola, Calle & Schachter (1960). By measuring the diameter of the region of blue dye accumulation at the injection site on the flank in both untreated control animals and in animals pretreated with various drugs, the degree of inhibition of the response afforded by the drug was determined.

Materials. Bradykinin was a synthetic nonapeptide (Nicolaides & DeWald, 1961) kindly supplied by Dr Collier in the form of a crystalline solid of approximately 80% purity. This peptide is now known to be identical with natural bradykinin (Shorley & Collier, 1960; Lewis, 1960b; Konzett & Sturmer, 1960b).

Kallidin was prepared from an incubated mixture of ox serum, which had been heated to 56° C for 3 hr, and human saliva, according to the method of Holdstock, Mathias & Schachter (1957).

The urinary kinin was prepared from a pooled sample of human urine by *n*-butanol extraction as described by Gaddum & Horton (1959).

Substance P was prepared by extraction of acetone-dried and *n*-butanol-washed ox brain with aqueous acid according to the general method of Amin, Crawford & Gaddum (1954) as subsequently modified by Leach (1959).

Salivary kallikrein for these experiments was obtained from human saliva, diluted at the time of use with 0.9% saline solution.

Serum kallikrein was obtained from guinea-pig or ox serum, which had been dialysed in cellophane tubing against distilled water for 48 hr at 4° C, and stored at this temperature until it was diluted for use with Tyrode solution at 37° C. In the case of both guinea-pig and ox serum maximum activation of the enzyme was obtained by a 1:15 dilution.

Histamine diphosphate, acetylcholine bromide and 5-hydroxytryptamine creatinine sulphate were used, and the doses refer to free base in each case. Sodium salicylate, acetylsalicylic acid, 2:6-dihydroxybenzoic acid, sodium α-4-*sec*-butylphenoxypropionate and phenylbutazone were used as freshly prepared solutions adjusted to neutrality (*p*H 7) with sodium hydroxide.

RESULTS

The inhibition of kinin formation by analgesic-antipyretic drugs. When guinea-pig or ox serum was diluted 1:15 with Tyrode solution and incubated for 5 min at 37° C, a smooth-muscle-stimulating kinin was produced. When diluted serum was added to an organ bath containing a strip of isolated guinea-pig ileum it caused a slightly delayed slow contraction. As little as 0.4 ml. of the diluted serum usually produced a maximal contraction. The undiluted serum, when added to the bath directly, produced no contraction for at least 2 min. If the contact time was extended beyond this, a very slow contraction occurs which reaches a maximum in about 6 min. The incubation of the diluted serum was then performed in the presence of 100 μg/ml. 2:6-dihydroxybenzoic acid, and when the incubated mixture was added to the organ bath the response was reduced to 15% of that obtained in the control. When the incubation of the diluted serum was performed in the absence of 2:6-dihydroxybenzoic acid, but an equivalent amount was added to the organ bath 1 min prior to the addition of the incubated and diluted serum, however, the response was the same as in the control, as shown in Fig. 1. This indicates that the inhibitor does not alter the responsiveness of the gut to the kinin when it is formed, but rather it inhibits its formation from kallidinogen by the enzyme kallikrein, which is activated by the dilution of the serum.

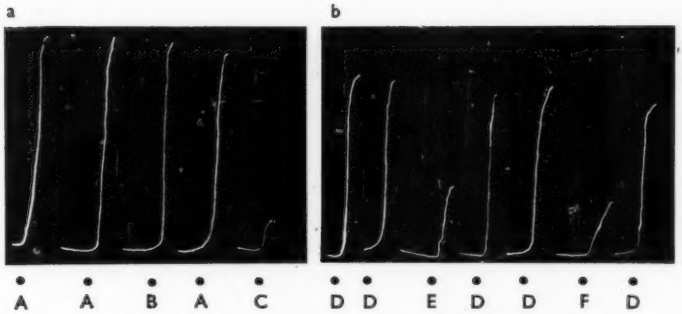

Fig. 1. Effect of analgesic-antipyretic drugs on formation of kallidin. Contractions of isolated guinea-pig ileum to 0.2 ml. incubated 1:15 dilution of guinea-pig serum at (A); to 20 μg 2:6-dihydroxybenzoic acid at (B); to 0.2 ml. 1:15 dilution guinea-pig serum incubated in presence of 100 μg/ml. 2:6-dihydroxybenzoic acid at (C); to 0.1 ml. incubated mixture of heated ox serum and salivary kallikrein at (D); to 0.1 ml. mixture of heated ox serum and salivary kallikrein incubated in the presence of 10 μg sodium salicylate at (E); to 0.1 ml. mixture of heated ox serum and salivary kallikrein incubated in presence of 10 μg phenylbutazone at (F). Contact time—60 sec.

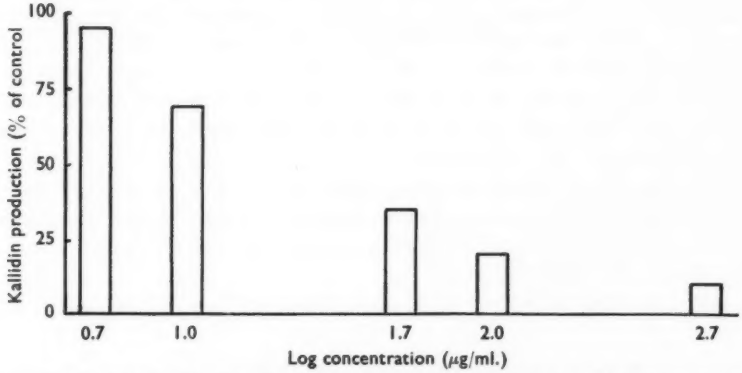

Fig. 2. The inhibition of kallidin formation by serum kallikrein, using fresh dialysed ox serum, in the presence of various concentrations of 2:6-dihydroxybenzoic acid, diluted 1:15 with Tyrode solution, and incubated for 5 min at 37° C. The production of kallidin will be approximately proportional to the antilogarithm$_{10}$ of the height of contraction of the gut.

Fig. 2 gives the results of experiments to determine the degree of inhibition of ox serum kallikrein, using various concentrations of 2:6-dihydroxybenzoic acid. Table 1 gives the concentrations of various inhibitors which were needed to reduce the kinin formation by 50% in the case of ox serum and human salivary kallikrein. Essentially similar results were obtained with guinea-pig serum.

Ox serum which had been heated to 56° C for 3 hr was incubated for 5 min at 37° C with 10 times its volume of human salivary kallikrein solution (final concentration equivalent to saliva diluted 1:100). A smooth-muscle-stimulating kinin was formed which caused an isolated segment of guinea-pig ileum to contract slowly,

TABLE 1

INHIBITION OF KALLIDIN FORMATION IN THE PRESENCE OF ANALGESIC-ANTIPYRETIC DRUGS

The inhibitors were incubated with the enzyme-substrate mixture in the final concentration shown. The incubated mixtures were added to a segment of guinea-pig ileum in an isolated organ bath and the heights of contraction recorded. The amount of kallidin formed is proportional to the antilogarithm$_{10}$ of the height of contraction, in the range of doses used in this experiment

Inhibitor	Concentration required to inhibit kinin formation by 50%, in μg/ml.	
	Ox serum kallikrein Mean±s.e.	Human salivary kallikrein Mean±s.e.
Salicylate	116±13	121±15
Aspirin	87±9	89±8
2:6-Dihydroxybenzoic acid	39±7	47±8
Sodium α-4-*sec*-butylphenoxy-propionate	74±10	82±11
Phenylbutazone	29±6	34±5

after a slight delay. Approximately 0.2 ml. of the incubated mixture usually produced a maximal contraction. The kallikrein solution and the heated serum were without significant effect when added to the bath separately. When the incubation was performed in the presence of various analgesic-antipyretic drugs the kinin was not formed so rapidly, and the concentrations of these inhibitors required were very similar to those given in Table 1. The possibility that the responsiveness of the gut to the kinin was altered was excluded by adding an equivalent amount of each inhibitor to the organ bath 1 min prior to the addition of the kinin containing mixture ; the response was not affected.

In the concentrations needed to inhibit kinin formation by 50%, the analgesic-antipyretic drugs tested in these experiments did not affect the response of the isolated guinea-pig ileum to synthetic bradykinin, substance P, urinary kinin, histamine, acetylcholine or 5-hydroxytryptamine.

The effect of these inhibitors on other kinin-forming enzyme systems is being investigated.

Antagonism between analgesic-antipyretic drugs and the vasodepressor effect of various substances in the dog. Fig. 3 shows the results of a typical experiment, where several substances which produce a fall in blood pressure were administered intravenously to an anaesthetized dog before and after an infusion of a moderately high dose of one of the analgesic-antipyretic drugs. The fall in blood pressure produced by bradykinin, kallidin, histamine, acetylcholine and 5-hydroxytryptamine was either unaffected or very slightly reduced, whereas that produced by human salivary kallikrein was abolished. In some experiments the response to salivary kallikrein was a sharp fall in blood pressure, a partial return to the baseline, and then a very protracted and sometimes incomplete return to the original value. In this case the antagonists would not only reduce the main fall in blood pressure but also hasten the return to the baseline blood pressure. This confirms a preliminary communication of Guth (1960), who has shown that salicylates inhibit the vasodepressor effect of salivary kallikrein. Of the five antagonists tested, salicylate, acetylsalicylate, α-4-*sec*-butylphenoxypropionate, 2:6-dihydroxybenzoic acid and

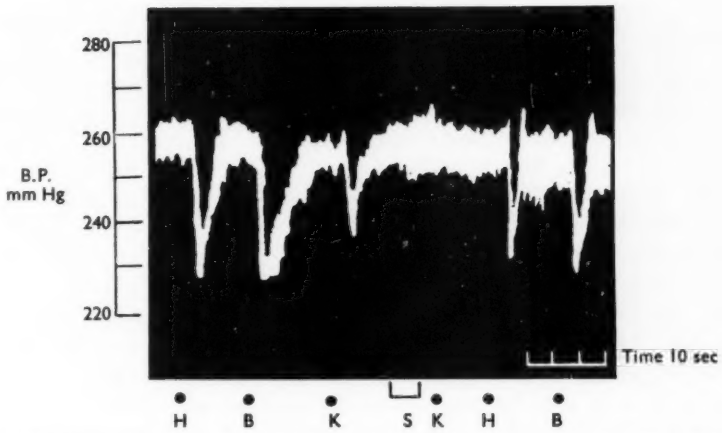

Fig. 3. The antagonism shown by sodium salicylate towards the vasodepressor effect of histamine, bradykinin and human salivary kallikrein. Carotid blood pressure of a 5 kg female dog, anaesthetized with chloralose. H, histamine 4 μg; B, bradykinin 8 μg; K, human salivary kallikrein, equivalent to 0.08 ml. saliva; S, sodium salicylate, 200 mg/kg.

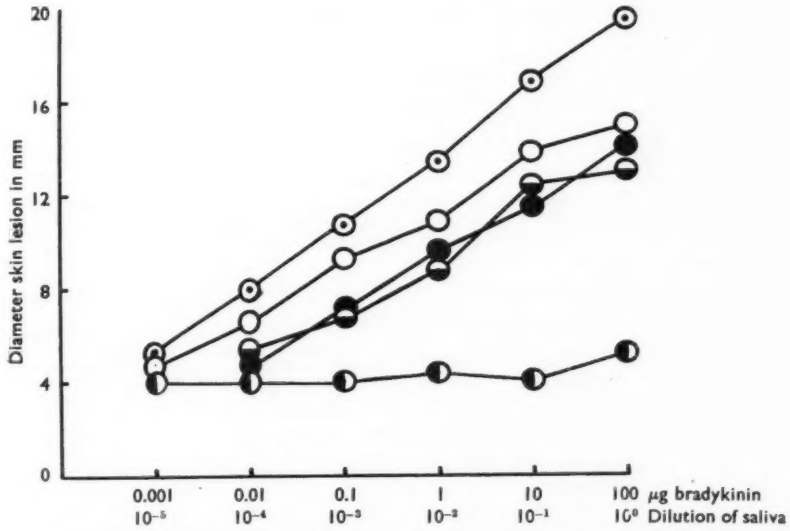

Fig. 4. The antagonism between intravenously administered salicylate and 2 : 6-dihydroxybenzoic acid and the capillary permeability enhancing effect of intradermally injected human salivary kallikrein and bradykinin respectively, in the rabbit. ⊙ —— ⊙, Control rabbits, given normal saline and injected with kallikrein; ○—— ○, given sodium salicylate 100 mg/kg and injected with kallikrein; ◐ —— ◐, given 2:6-dihydroxybenzoic acid 50 mg/kg and injected with kallikrein; ● —— ●, given 2:6-hydroxybenzoic acid 50 mg/kg and injected with bradykinin; ◒ ——◒, given normal saline and injected with bradykinin.

phenylbutazone, the last two were the most potent, being effective in doses of 50 mg/kg; the others required at least 150 mg/kg to produce a comparable inhibition.

The antagonism between analgesic-antipyretic drugs and the vasodepressor effects of salivary kallikrein were manifest for only a short time, and within 45 min of the injection of 2:6-dihydroxybenzoic acid, for example, the response to kallikrein was already back to control values. Since the effect was so short-lived, it was thought possible that the mode of action involved adrenal medullary stimulation. To test this possibility the experiment was repeated in a dog which had both adrenal glands excluded prior to the experiment; 2:6-dihydroxybenzoic acid still inhibited the fall in blood pressure produced by salivary kallikrein.

Antagonism between analgesic-antipyretic drugs and the accumulation of protein-bound dye at the site of injection of kallikrein. Bradykinin injected intradermally into rabbits causes the accumulation of circulating protein-bound Evans blue at the site of injection. We have used synthetic bradykinin and confirmed the findings of Bhoola, Calle & Schachter (1960) that the slope of the curve relating relative diameter of skin blueing to intradermal dose of bradykinin is flat. With human salivary kallikrein there is also a flat dose-response curve (Fig. 4). Rabbits which had been given intravenous infusions of various analgesic-antipyretic drugs were also tested, and it was found that, whilst the response to injected salivary kallikrein was very nearly abolished, the response to injected bradykinin was only very slightly affected.

Table 2 gives the doses of various analgesic-antipyretic drugs which are required to inhibit the response to injected salivary kallikrein by 50% in the dog and in the rabbit.

TABLE 2

INHIBITORS OF THE VASODEPRESSOR AND CAPILLARY PERMEABILITY ENHANCING ACTION OF HUMAN SALIVARY KALLIKREIN, IN THE DOG AND IN THE RABBIT RESPECTIVELY

	Dose of antagonist (mg/kg intravenously) required to inhibit response by 50%	
Antagonist	Vasodepressor effect Mean±s.e.	Capillary hyperpermeability Mean±s.e.
Salicylate	181±32	192±28
Aspirin	153±22	174±25
2:6-Dihydroxybenzoic acid	87±19	95±14
Sodium α-4-*sec*-butylphenoxy-propionate	119±20	113±18
Phenylbutazone	76±12	79±13

The inhibition of the action of kallikrein in the rabbit was manifest within 5 min of the intravenous injection of the analgesic-antipyretic drug, and lasted for several hours, as shown in Fig. 5. We are not able to explain the longer duration of action of these antagonists in the rabbit than in the dog.

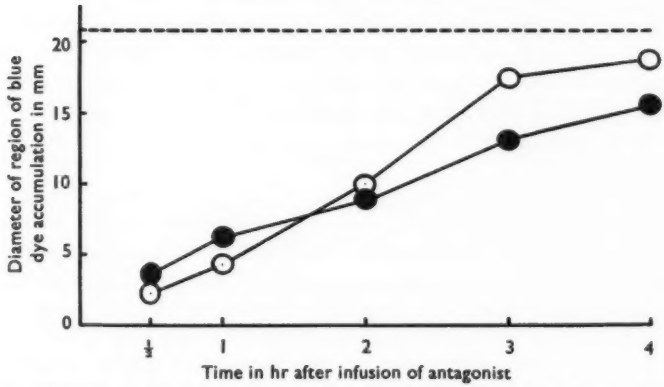

Fig. 5. The inhibition of the capillary permeability enhancing effect of intradermally injected human salivary kallikrein in the rabbit, using 2: 6-dihydroxybenzoic acid and acetylsalicylate, administered as a single dose intravenously. ○ —— ○, 2: 6-dihydroxybenzoic acid, 50 mg/kg; ● —— ●, acetylsalicylate, 200 mg/kg. The horizontal line marked – – – – denotes the mean response of untreated control animals. The dose of salivary kallikrein injected was 0.1 ml. of a 1 : 100 dilution of saliva.

DISCUSSION

There is no conclusive evidence available at the moment to indicate the nature of the mediator of the inflammatory response. It is possible that different types of inflammatory stimuli, in different species and even at different times during an inflammatory reaction, involve different mediators. There is considerable evidence, however, that some of the products of protein degradation are involved in the process, and the subject has been reviewed recently by Lewis (1960a) from the point of view of the part which plasma kinins play in these reactions. Spector (1958) has reviewed the factors which control capillary permeability and the way in which endogenous proteolytic enzymes exert their effect.

The implication of a particular mediator in the inflammatory reaction places upon the investigator the responsibility for demonstrating several crucial facts. For example, it is necessary to show that the mediator actually occurs in the damaged tissues during an inflammatory reaction. The mediator should also be shown not to occur in the tissues, at any rate in an active form, at other times. The administration of exogenous mediator should cause a reaction closely simulating inflammatory reactions elicited in other ways. Antagonists of the inflammatory response to this mediator should also be capable of inhibiting the effects of other inflammatory stimuli. Plasma kinin can fulfil some of these criteria at the moment.

There is no direct evidence that the liberation of bradykinin occurs in inflammatory reactions. However, Elliott, Horton & Lewis (1960) with the pure natural bradykinin which they prepared from ox blood, and Konzett & Sturmer (1960a) with a synthetic peptide of the same structure, indicate that not only is pure bradykinin capable of producing many of the typical symptoms of inflammation but that in some species it is one of the most potent substances known in this respect.

In this paper we have attempted to bring forward some evidence in favour of the theory (Hilton & Lewis, 1955) that the endogenous liberation of plasma kinin is an important step in the mediation of the inflammatory reaction, at any rate under certain conditions. The compounds which we have tested belong to a chemically heterogenous group of substances with the ability to inhibit certain types of inflammatory reaction, as well as certain enzyme systems which are known to produce plasma kinin.

One would expect that in general the more potent anti-inflammatory agent should also be the more potent inhibitor of the kinin-forming enzyme system, if the kinin is in fact involved in the inflammation. This has proved to be the case ; the two most potent inhibitors of kallikrein in our experiments, namely, phenylbutazone and 2:6-dihydroxybenzoic acid, have both been shown to be more potent than, for example, salicylates in the treatment of rheumatoid diseases of man. Reid, Watson, Cochran & Sproull (1951) demonstrated the considerably greater potency of 2:6-dihydroxybenzoic acid compared with salicylate in acute rheumatic fever in man. Similarly, Buttle (1951) has shown that 2:6-dihydroxybenzoic acid is more potent than salicylate in preventing formaldehyde-induced foot swelling in mice. Against this evidence, however, is the fact that Adams & Cobb (1958) showed that 2:6-dihydroxybenzoic acid was considerably less potent than salicylate in inhibiting the ultra-violet radiation induced erythema in the skin of guinea-pigs. Phenylbutazone is considered to be among the most potent anti-rheumatoid drugs at present in therapeutic use.

The concentrations of analgesic-antipyretic drugs which we have found necessary to inhibit kallikrein bear a similarity to the blood levels of these substances which Goodman & Gilman (1955) have reported to be necessary for a significant anti-rheumatoid effect in man (approximately 350 μg/ml. in the case of salicylate). The present series of experiments do not exclude the possibility that a significant part of the anti-inflammatory action of the analgesic-antipyretic drugs is caused by the inhibition of plasma kinin rather than by the inhibition of the plasma kinin-forming enzymes. Other workers have previously shown that various pharmacological actions of bradykinin can be inhibited by analgesic-antipyretic drugs. Thus Collier & Shorley (1960) have demonstrated a specific antagonism between the broncho-constrictor action of bradykinin and various salicylates in the anaesthetized guinea-pig, using doses appreciably less than were found to be necessary, for example, in our experiments with dogs and rabbits. It has even been claimed that in very high doses salicylate will inhibit the contraction of the isolated guinea-pig ileum to histamine (Mongar & Schild, 1957). Lecomte (1960) has shown an antagonism between large doses of phenylbutazone and the cardiovascular and capillary permeability enhancing actions of bradykinin in the rabbit. In our experience, however, based upon the use of three independent test preparations, the evidence seems to indicate that the inhibition of the kinin-forming enzyme systems takes place in a lower concentration of analgesic-antipyretic drug than is required to inhibit the actions of the plasma kinin itself, and hence may more probably explain the anti-inflammatory action of this class of compounds.

We wish to thank the Rockefeller Foundation for a generous grant towards the expenses of this work, and Dr Main, Dr Adams and Dr Cobb, of Boots Pure Drug Company, for

supplies of sodium α-4-*sec*-butylphenoxypropionate. We are also very grateful to Dr H. O. J. Collier, of Parke, Davis & Co., for providing us with a sample of pure synthetic bradykinin.

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THE NEUROMUSCULAR BLOCKING ACTION OF γ-OXALOLAUDONIUM BROMIDE

BY

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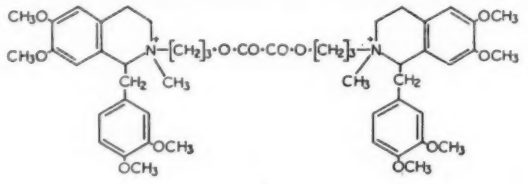
In small animals γ-oxalolaudonium caused flaccid paralysis; in the cat it produced a curare-like rather than a decamethonium-like block of neuromuscular transmission. The potency of γ-oxalolaudonium was only 1/30 to 1/40 that of suxamethonium, but the duration of paralysis was very short, being about one-half that of equiactive doses of suxamethonium. Successive doses of γ-oxalolaudonium were not cumulative and the paralysis could be antagonized by neostigmine. γ-Oxalolaudonium exhibited low toxicity especially in artificially ventilated animals, and it did not show ganglion-blocking or histamine-releasing activity to any large degree.

Laudexium is a synthetic drug whose mode and duration of action closely resemble those of tubocurarine. Suxamethonium is a neuromuscular blocking agent of brief duration which acts by depolarizing the motor end-plate region. It was thought that the brief duration of suxamethonium and the curare-like mode of action of laudexium might be combined in one compound by incorporating some



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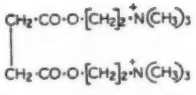

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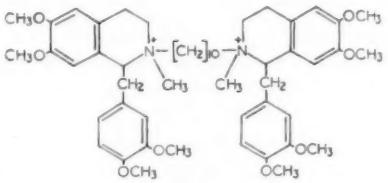

III

of the chemical features of suxamethonium into the laudexium molecule. Three series of compounds—symmetrical bis-esters, unsymmetrical bis-esters and symmetrical bis-amides—were therefore synthesized (Collier, Gladych, Macauley

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& Taylor, 1958a & b ; Gladych & Taylor, 1961). From the initial pharmacological work on these compounds already reported (Collier *et al.*, 1958a & b), one symmetrical bis-ester appeared to be worthy of further examination, and it is the more detailed pharmacology of this compound, designated γ-oxalolaudonium, that is now described. The chemical structure of γ-oxalolaudonium (I) and its relationship to suxamethonium (II) and laudexium (III) are shown above.

METHODS

Drugs. The following salts were used: γ-oxalolaudonium bromide, suxamethonium chloride, tubocurarine chloride, neostigmine methylsulphate, nicotine tartrate and histamine acid phosphate. Drugs were dissolved in normal saline and doses are given as weights of their respective salts.

Action in mice. Paralysing activity was determined in male albino mice weighing 18 to 22 g using the rotating drum technique of Collier, Hall & Fieller (1949). In acute toxicity tests deaths were recorded after 7 days.

Action in rabbits. Paralysis was determined by loss of righting reflex in white Himalayan rabbits of either sex and of body weight 2 to 3 kg. In acute toxicity tests deaths were recorded after 7 days. Blood pressure was recorded by mercury manometer from the carotid artery of rabbits anaesthetized with urethane (1 g/kg intravenously). Drugs were administered intravenously through a cannula in the jugular vein.

Action in chicks. Drugs were administered intravenously by the jugular vein to day-old chicks.

Action on the myoneural junction. In vitro experiments were performed on the isolated rectus abdominis muscle of the frog according to the procedure of Brittain, Chesher, Collier & Grimshaw (1959). *In vivo* experiments were performed on cats anaesthetized with chloralose (80 mg/kg intraperitoneally). A hind limb was set up in a vertical position on a Brown–Schuster myograph stand. Shielded electrodes were placed on the sciatic nerve and the nerve was ligated central to the electrodes. Twitches and tetani of the tibialis anterior muscle were elicited by supramaximal rectangular pulses of 0.35 to 1.0 msec duration. The muscle was attached to a flat spring myograph and the contractions were recorded on smoked paper. Blood pressure was recorded from the carotid artery with a mercury manometer. While the animal was artificially ventilated, drugs were injected intravenously through a cannula in either the femoral or jugular vein.

Action on ganglia. The ganglionic blocking activity of drugs was investigated using the isolated ileum of the guinea-pig by the method of Feldberg (1951).

Release of histamine. Liberation of histamine was assessed by weal formation in human skin (Collier & Macauley, 1952).

RESULTS

Action in mice, rabbits and chicks

γ-Oxalolaudonium when administered intravenously to mice in effective doses (1 to 2 mg/kg) caused a short-lasting flaccid paralysis neither preceded nor followed by excitation. The results of these experiments together with studies on the intravenous acute toxicity of γ-oxalolaudonium are summarized in Table 1. Information

TABLE 1

PARALYSING ACTIVITY AND ACUTE TOXICITY OF γ-OXALOLAUDONIUM BROMIDE INJECTED INTRAVENOUSLY INTO MICE

Index	No. of experiments	Mean value mg/kg	Range of values mg/kg
ED50	10	1·61	1·38–1·95
LD50	6	5·31	4·76–6·46

from experiments of this type led to the belief that the drug might be unstable in aqueous solution. A 0.5% solution of γ-oxalolaudonium was prepared in saline, and suitable dilutions were administered intravenously to mice immediately, 24 hr and 96 hr after preparation. The paralysing activity and acute toxicity of γ-oxalolaudonium were determined, and the results of these experiments, summarized in Table 2, show that the drug was unstable in aqueous solution, and therefore in all other experiments solutions of γ-oxalolaudonium were freshly prepared.

TABLE 2

INSTABILITY OF γ-OXALOLAUDONIUM BROMIDE IN AQUEOUS SOLUTION

Time in hr after preparation of solution	No. of mice	ED50±s.e. mg/kg	LD50±s.e. mg/kg
0	45	1·4±0·06	5·5±0·04
24	24	4·0±0·15	—
96	36	9·3±0·70	22·4±0·80

In rabbits, intravenous γ-oxalolaudonium (1 to 2 mg/kg) caused immediate loss of the righting reflex which lasted from 2 to 4 min and was associated with respiratory difficulty. After recovery of the righting reflex the head-drop posture was evident for a very short time. The intravenous LD50 of the drug lay between 2 and 5 mg/kg.

The mode of action of γ-oxalolaudonium was investigated in chicks. While intravenous suxamethonium, 0.05 mg/kg, caused rigid extension of the legs and retraction of the head, intravenous γ-oxalolaudonium, 12.5 mg/kg and above, caused a flaccid paralysis similar to that described for tubocurarine by Buttle & Zaimis (1949).

Action on myoneural junction

In vitro *experiments.* γ-Oxalolaudonium, 1.33×10^{-6}, failed to stimulate the isolated frog rectus abdominis preparation but antagonized its response to suxamethonium.

In vivo *experiments.* In the cat, γ-oxalolaudonium, 3 to 4 mg/kg intravenously, caused 90 to 100% paralysis of the indirectly evoked maximal twitches of the tibialis anterior muscle without initial stimulation. These experiments, which are summarized in Table 3, show that the drug has a short-lasting neuromuscular

TABLE 3

PARALYSING ACTIVITY OF γ-OXALOLAUDONIUM ON THE MAXIMAL TWITCHES OF THE CAT TIBIALIS ANTERIOR MUSCLE IN RESPONSE TO INDIRECT STIMULATION

Duration is expressed as the time between treatment and recovery of the twitch tension to 75% of its value before treatment

Dose of γ-oxalolaudonium bromide mg/kg	1·0	2·0	3·0	4·0
No. of experiments	3	1	14	2
Mean % reduction of twitch response±s.e.	13·0±6·1	25·5	91·0±2·2	100
Mean duration of effect (min)±s.e.	—	1·1	3·3±0·2	7·5±0·2

blocking action. Experiments in which γ-oxalolaudonium was compared with suxamethonium showed that γ-oxalolaudonium had only 1/30 to 1/40 of the potency of suxamethonium, although its duration was very short, being only about one-half that of equiactive doses of suxamethonium (Fig. 1). Moreover, successive equal

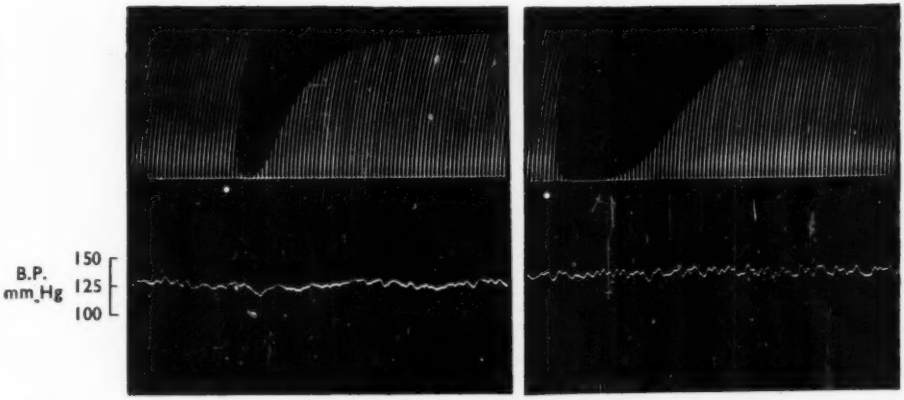

Fig. 1. Cat, chloralose. Upper tracing, maximal twitches of the tibialis anterior muscle elicited by indirect stimulation. Lower tracing, carotid blood pressure. Time scale, 10 sec intervals. Left-hand panel, 3 mg/kg γ-oxalolaudonium; right-hand panel, 150 μg/kg suxamethonium; both drugs injected intravenously.

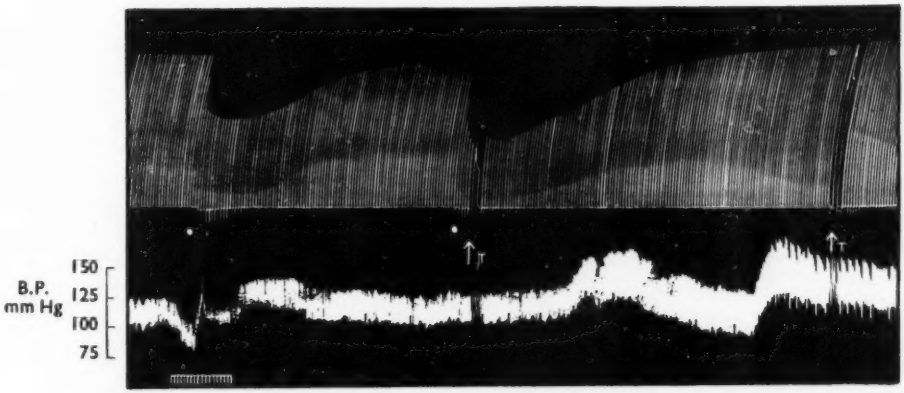

Fig. 2. Cat, chloralose. Upper tracing, maximal twitches of the tibialis anterior muscle elicited by indirect stimulation. Lower tracing, carotid blood pressure. Time scale, 10 sec intervals. At dots 50 μg/kg suxamethonium injected intravenously. ↑T indicates tetanus of 10 sec duration and frequency 30/sec.

doses of γ-oxalolaudonium did not last longer in effect than the initial dose, although successive equal doses of suxamethonium showed cumulative effects.

The response of the muscle to tetanic stimulation of the motor nerve has been used as an indication of the mode of action of neuromuscular blocking substances (Paton & Zaimis, 1949). During partial paralysis by suxamethonium, tetanic tension was fairly well sustained (Fig. 2); following partial paralysis by γ-oxalolaudonium, however, tetanic stimulation produced only a poorly sustained response (Fig. 3).

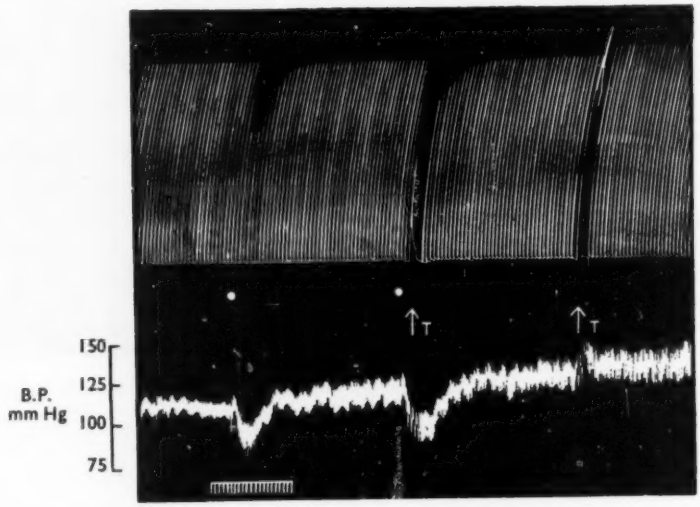

Fig. 3. Cat, chloralose. Upper tracing, maximal twitches of the tibialis anterior muscle elicited by indirect stimulation. Lower tracing, carotid blood pressure. Time scale, 10 sec intervals. At dots 2 mg/kg γ-oxalolaudonium injected intravenously. ↑T indicates tetanus of 10 sec duration and frequency 30/sec.

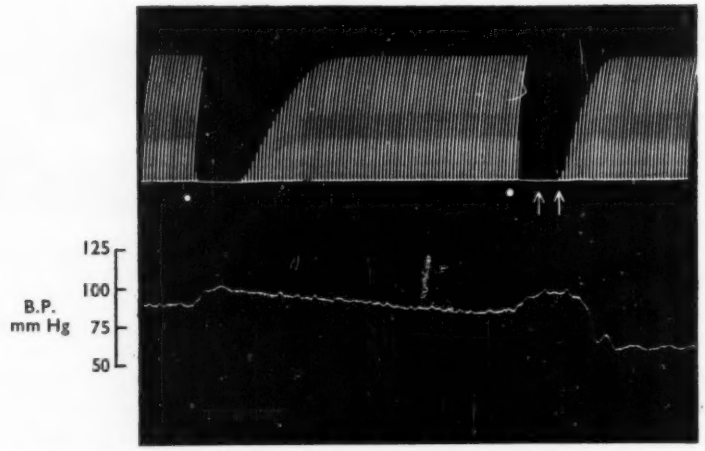

Fig. 4. Cat, chloralose. Upper tracing, maximal twitches of the tibialis anterior muscle elicited by indirect stimulation. Lower tracing, carotid blood pressure. Time scale, 10 sec intervals. At dots 10 mg/kg γ-oxalolaudonium, at arrows 30 μg/kg neostigmine. Both drugs injected intravenously.

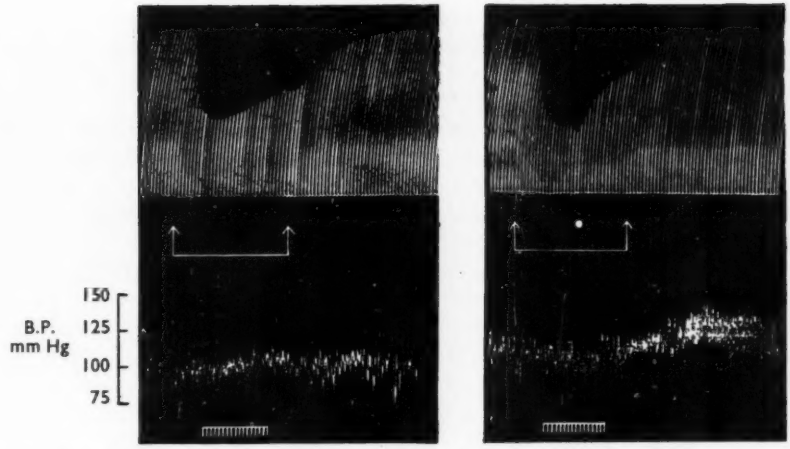

Fig. 5. Cat, chloralose. Upper tracing, maximal twitches of the tibialis anterior muscle elicited by indirect stimulation. Lower tracing, carotid blood pressure. Time scale, 10 sec intervals. During the period marked by arrows and horizontal bar a 1% solution of γ-oxalolaudonium was infused intravenously 0.5 ml./min. At dot, 20 μg/kg neostigmine was injected intravenously.

Since the results so far described indicated that γ-oxalolaudonium produced a curare-like block of neuromuscular transmission, further experiments were carried out to confirm this indication in two ways. In one, neostigmine was administered after complete block by γ-oxalolaudonium (Fig. 4); in the alternative method, neostigmine was administered during a partial paralysis induced by a constant infusion of γ-oxalolaudonium (Fig. 5). It is evident from these experiments that neostigmine antagonized the neuromuscular block induced by γ-oxalolaudonium.

Acute toxicity in artificially ventilated animals

Since the predominant toxic effect of γ-oxalolaudonium in normal animals was respiratory arrest, the toxicity of γ-oxalolaudonium was studied in artificially ventilated animals. Three rabbits tolerated successive intravenous doses of 5, 10 and 20 mg/kg, but of these only one animal survived a further dose of 40 mg/kg. Of three cats, two survived an intravenous dose of 40 mg/kg γ-oxalolaudonium. In

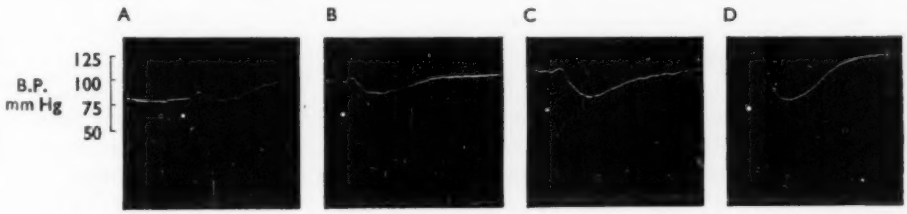

Fig. 6. Rabbit, urethane. Carotid blood pressure. Time scale, 10 sec intervals. At A, B, C and D, γ-oxalolaudonium injected intravenously at doses of 5, 10, 20 and 40 mg/kg respectively.

both rabbits and cats, 10 mg/kg and above produced a temporary fall in blood pressure, the results of one such experiment in the rabbit being illustrated in Fig. 6.

Action on ganglia

In view of the hypotensive action of very large intravenous doses of γ-oxalolaudonium in anaesthetized animals, the drug was examined for ganglionic blocking activity. The responses of the isolated guinea-pig ileum to nicotine were compared in the absence or in the presence of γ-oxalolaudonium. In a concentration of 2.5×10^{-5}, γ-oxalolaudonium did not increase muscle tone, nor did it induce spontaneous movements of the tissue, nor modify the response of the preparation to nicotine. In contrast, tubocurarine in a concentration of 4×10^{-6} reduced the nicotine response by 49% (Fig. 7). It is evident from this experiment that γ-oxalolaudonium did not exhibit a ganglionic blocking action on this preparation at the largest dose tested.

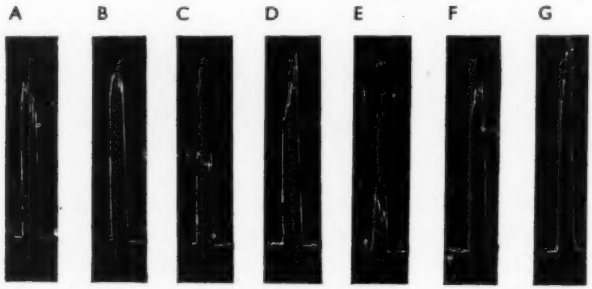

Fig. 7. Isolated guinea-pig ileum in 20 ml. bath. Contractions in response to 40 μg of nicotine. At A and G, no treatment; at B, D and F, 80, 160 and 500 μg γ-oxalolaudonium respectively; at C and E, 80 and 160 μg of tubocurarine respectively. Four histamine responses between each nicotine effect have been omitted from the tracing.

Release of histamine

As tubocurarine and laudexium are known to release histamine, the possible effect of γ-oxalolaudonium in this respect was investigated in human subjects. In two subjects, intradermal injection of 100 μg γ-oxalolaudonium caused local weal and flare attributed to release of histamine. Reference to previous skin tests with established neuromuscular blocking agents (Collier & Macauley, 1952) shows that γ-oxalolaudonium had less than half the potency in releasing histamine of laudexium, which in turn is less active than tubocurarine.

DISCUSSION

The experiments described show that γ-oxalolaudonium caused a very brief paralysis in chicks, mice, rabbits and cats. Five facts indicate that its mode of action resembles that of tubocurarine and differs from that of decamethonium or suxamethonium: (1) γ-oxalolaudonium did not stimulate the isolated rectus abdominis of the frog, but antagonized the action of suxamethonium on this preparation; (2) it did not produce muscle fasciculations nor potentiate the maximal twitch

of the tibialis ; (3) during partial paralysis by γ-oxalolaudonium, tetanic stimulation produced a poorly sustained contracture ; (4) neostigmine antagonized the neuromuscular blockade due to γ-oxalolaudonium ; and (5) in chicks, γ-oxalolaudonium produced a flaccid paralysis.

In γ-oxalolaudonium the large heterocyclic end-groups of laudexium are linked by an ester chain somewhat resembling that of suxamethonium. The brevity and curare-like action of γ-oxalolaudonium show that, by combining some of the chemical features of laudexium and of suxamethonium, a compound can be obtained having some of the desirable features of each drug. Unfortunately this combination was attended by a marked loss of potency, which renders doubtful the clinical value of γ-oxalolaudonium.

We wish to thank Miss B. Macauley for some experimental contributions and Mr D. Rowe for technical assistance. We are also indebted to the directors of Allen & Hanburys for permission to publish this work.

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PERIPHERAL ACTIONS OF HEXAMETHONIUM IN RELATION TO THE DECREASING EFFECTS OF REPEATED DOSES ON THE BLOOD PRESSURE OF ANAESTHETIZED RATS

BY

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(*Received June* 2, 1961)

When hexamethonium was given intravenously to anaesthetized rats, the depressor response produced by successive doses became less. This effect was not related to the duration of anaesthesia and the possible recovery of cardiovascular reflex activity. When the rat's own blood was perfused at constant rate through one hind limb, successive doses of hexamethonium reduced the hind-limb perfusion pressure to the same extent each time, but their effect on the blood pressure of the remainder of the animal became less. When the hind limb was perfused at constant rate with blood from a separate donor rat, hexamethonium in the donor blood produced a very small increase in the vasoconstrictor response to noradrenaline in the innervated hind limb but not in the denervated hind limb.

Evidence that hexamethonium and pentolinium can sensitize the blood vessels of the hind limb of the cat to the constrictor effects of adrenaline and noradrenaline has been given by Zaimis (1955, 1956) and by Mantegazza, Tyler & Zaimis (1958). They have suggested that this sensitizing action might explain (1) why hexamethonium brings about potentiation of the pressor effects of adrenaline and noradrenaline in the whole animal, (2) why the blood pressure response to repeated doses of hexamethonium becomes less in anaesthetized animals, and (3) why "tolerance" develops to the blood pressure lowering action of many ganglion-blocking compounds in man.

In the following experiments, the modification of the depressor response to successive doses of hexamethonium has been studied in the anaesthetized rat, and concurrent changes in the peripheral resistance of one of the hind limbs have been measured by a technique based on constant-rate blood-perfusion. The peripheral effect of hexamethonium on the sensitivity of blood-perfused denervated and innervated hind-limb blood vessels to noradrenaline has also been studied.

METHODS

Adult male albino rats (280 to 380 g) were used throughout. Arterial blood pressure was recorded by a small-volume mercury manometer connected to the femoral artery by a polythene tube filled with heparin-saline (250 u./ml.). In most experiments rats were anaesthetized with chloralose (50 to 60 mg/kg) injected intravenously. In one group, more prolonged

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anaesthesia was produced by injecting urethane (600 to 700 mg/kg) subcutaneously 15 min after the chloralose. The rats were kept warm by overhead electric lamps.

Hind-limb perfusion experiments

Perfusion with animal's own blood. In these experiments the hind limb was perfused through the femoral artery at constant rate with blood taken from the opposite femoral artery. The hind limb was either left intact—this ensured normal nervous vasomotor tone in the hind limb and minimal blood loss and surgical trauma—or, alternatively, the hind limb was isolated at a level just proximal to the third trochanter of the femur, except for the femoral vein connexion, which was retained. The femoral and sciatic nerves were kept intact, every care being taken to maintain as complete hind-limb nervous tone and haemostasis of the cut surfaces as possible. With these preparations, the effect of hexamethonium on the hind-limb "peripheral resistance," as indicated by the perfusion pressure, could be studied independently of the blood pressure of the remainder of the animal.

Perfusion with blood from donor. In these experiments, the hind limb with its vascular system isolated was perfused at constant rate with blood from a separate donor rat (Field & Laverty, 1958). In some experiments, the femoral and sciatic nerves were kept intact, so that central vasomotor control was retained; in other experiments, the hind limb was completely isolated by cutting the nerves. With these preparations, the peripheral effect of hexamethonium on the vascular response of the hind limb to noradrenaline could be measured.

The response to noradrenaline was measured as the rise in perfusion pressure induced by injecting noradrenaline hydrogen tartrate (equivalent to 0.01 μg base in 0.05 ml. of 0.9% sodium chloride solution) into the perfused hind limb. Records were obtained of at least two responses to noradrenaline given at 10 min intervals at the inflow to the perfused limb. Hexamethonium bromide was injected into the blood perfusing the hind limb or intravenously into the donor animal. In either case, the dose was large (3 to 5 mg) and corresponded to an initial concentration in the perfusing blood of approximately 100 to 150 μg/ml. In most experiments, hexamethonium was given twice. Hind-limb perfusion pressures were recorded immediately before each noradrenaline injection.

In all perfusion experiments, heparin was used as anticoagulant. As with the blood pressure, the hind-limb perfusion pressure was recorded with a small-volume mercury manometer. The constant-rate blood-perfusion pump has been described by Field, de Graaf & Wallis (1958).

RESULTS

Repeated doses of hexamethonium

When repeated intravenous doses of 0.5 mg of hexamethonium bromide were given to anaesthetized rats at intervals which allowed recovery of the blood pressure between each dose, successive doses usually produced less effect. In some cases, the fall in pressure was similar and the rate of recovery became more rapid with successive doses, but, most often, both the fall in pressure and the time for recovery became less (Fig. 1). These results are similar to those observed in the cat by Zaimis (1956) and Mantegazza, Tyler & Zaimis (1958).

Duration of anaesthesia. Since the diminishing effect of repeated doses of hexamethonium might be the result of some degree of recovery of cardiovascular reflex activity associated with a decrease in the depth of anaesthesia with time, the effect of hexamethonium injected early in an experiment (within 1 hr of anaesthesia) was compared with the effect of a first dose of hexamethonium injected when anaesthesia was noticeably lighter. With combined chloralose-urethane anaesthesia, the mean percentage fall in blood pressure produced by the first dose of hexamethonium was

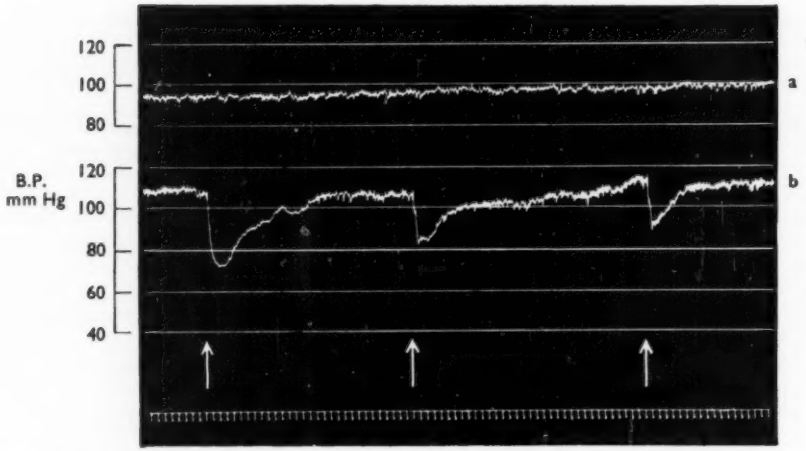

Fig. 1. Two rats, each 280 g, anaesthetized with chloralose-urethane. Simultaneous blood pressure records. (*a*) Control rat. (*b*) Decreasing blood pressure response to successive doses of hexamethonium bromide (0.5 mg) given at arrows. Time in min.

not appreciably different in the two groups. Thus in 7 rats in which 0.5 mg of hexamethonium bromide was first given within 30 to 60 min of anaesthesia, the mean fall of blood pressure was 26% (s.e. 2.6%) compared with 24% (s.e. 3.8%) in 6 rats which had not been given the first dose of hexamethonium till 3 to 4 hr later. In addition, the blood pressure response became less with repeated doses whether the hexamethonium was first given early or late.

Similarly, when chloralose alone was used as anaesthetic, 0.5 mg of hexamethonium bromide given within 30 to 40 min of anaesthesia produced a mean fall of blood pressure in 7 rats of 24% (s.e. 7.1%), which compared with the mean fall of 26% (s.e. 3.2%) observed when 0.5 mg of hexamethonium bromide was given to another 7 rats 1.5 to 2.5 hr after anaesthesia. As with chloralose-urethane anaesthesia, the blood pressure response became less in both groups when the hexamethonium dose was repeated, though it was noted in 3 cases that when the initial fall of blood pressure was small (less than 10%) the diminution of the response was small also.

Hind-limb perfusion experiments

These experiments were designed to estimate the extent to which any local vascular effect of hexamethonium might lead to a decreased blood pressure response to subsequent doses.

In experiments in which the intact hind limb of the rat was perfused at constant rate with its own blood, successive doses of hexamethonium bromide (0.5 mg) produced diminishing blood pressure responses, but in the hind limb the perfusion pressure was reduced by the same proportion each time (Fig. 2). When larger doses of hexamethonium (for example, 0.75 mg) were given, the same effects were observed. However, the recovery of the perfusion pressure after each response was

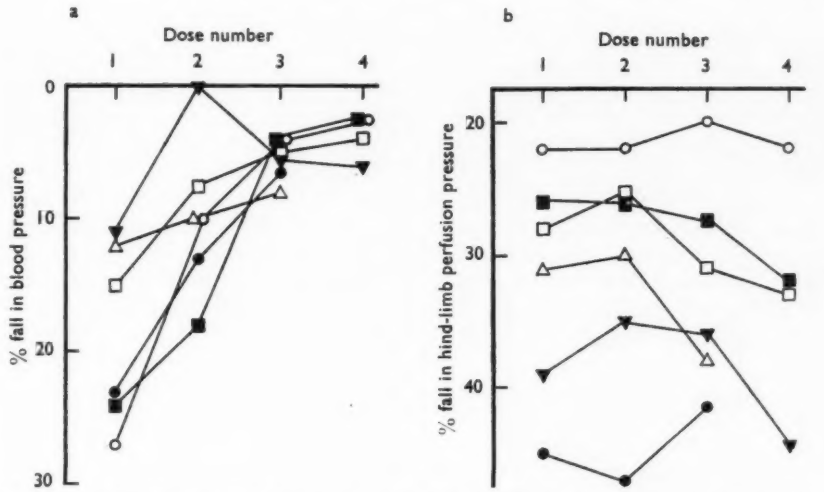

Fig. 2. Changes (*a*) in the blood pressure and (*b*) in the hind-limb perfusion pressure produced by successive doses of hexamethonium bromide (0.5 mg) given to 6 rats. Chloralose anaesthesia. Intact hind limb of each rat perfused with rat's own blood. Doses repeated only when blood pressure had returned to starting level. Results for each rat are distinguished by similar symbols.

less complete so that after several doses of hexamethonium the perfusion pressure was considerably less than at the start of the experiment even though the blood pressure had returned to normal (Fig. 3).

In these experiments, the hind-limb vascular bed was not completely isolated, and the perfusion pressure may have been affected by changes in pressure at the venous outflow or by changes in any collateral circulation present in the hind limb. Further

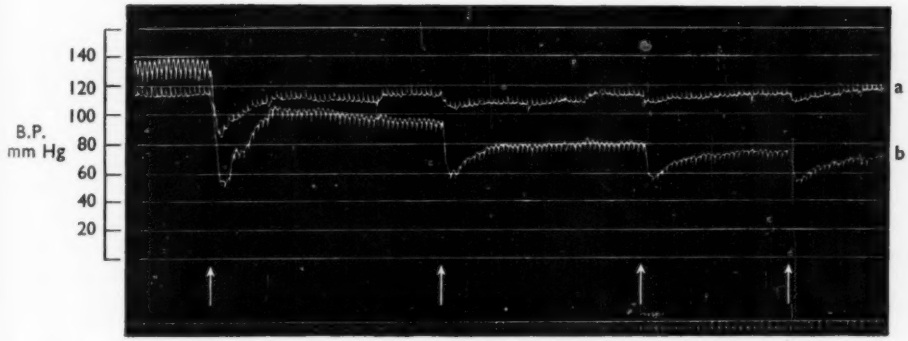

Fig. 3. Rat, 360 g, chloralose anaesthesia. Changes in (*a*) blood pressure and (*b*) hind-limb perfusion pressure produced by successive doses of hexamethonium bromide (0.75 mg) given intravenously at arrows. Hind limb perfused at constant rate with rat's own blood. Note cross-over of blood pressure and perfusion pressure records immediately after first arrow. Time in min.

experiments were therefore performed to see if these factors were important. In three experiments, all vascular connexions except the femoral vein were eliminated. Any collateral circulation was therefore removed. When repeated doses of hexamethonium were given, the changes in blood pressure and hind-limb perfusion pressure followed the same pattern as in the simpler preparation above. In another three experiments, all vascular connexions including the femoral vein were eliminated —only the nerve supply was left intact—and the hind limb was perfused with blood from a separate donor rat. When repeated doses of hexamethonium were given to the recipient animal, the blood pressure and hind-limb perfusion pressure responses showed similar patterns to those in the other experiments. These results suggest that the effects of any collateral circulation in the hind limb or of alterations in pressure at the venous outflow are not important.

Local vascular effects of hexamethonium

The above perfusion experiments suggest that hexamethonium has no local action on blood vessels large enough (at least in the hind limb) to produce a diminishing blood pressure response to repeated doses. However, hexamethonium may still have a small local action.

To obtain more direct evidence the effect of hexamethonium on the vasoconstrictor response to noradrenaline in the isolated hind limb perfused at constant rate with blood from a donor rat was measured. The results are given in Table 1. In the denervated hind limb there was no significant change in the response to noradrenaline

TABLE 1

EFFECT OF HEXAMETHONIUM BROMIDE ON PERFUSION PRESSURE AND VASOCONSTRICTOR RESPONSES TO NORADRENALINE IN THE ISOLATED HIND LIMB OF THE RAT

Test dose of 0.01 μg noradrenaline was added to the blood entering the hind limb. The hexamethonium was usually given to the donor animal. The control values in the last line were obtained in the same way as those of Expt. 2, but hexamethonium was not given

State of preparation	No. of preparations	Dose of hexamethonium (mg)	Mean perfusion pressure (mm Hg)		Mean % rise in perfusion pressure with noradrenaline (0.01 μg)	
			Before hexamethonium	After hexamethonium	Before hexamethonium	After hexamethonium
Denervated	9	3	61·5	63·1	52·0	51·8
Innervated (Expt. no. 1)	14	5	77·9	78·2	46·0	53·4
Innervated (Expt. no. 2)	10	5	112·8	114·4	44·5	50·0
Controls for Expt. no. 2	10	—	109·5	106·6	44·1	48·4

after hexamethonium, but in the innervated hind limb (Expt. 1) there was a significant increase ($0.02>P>0.01$, t test). In a second group of experiments using innervated hind limbs, control responses to noradrenaline were obtained during 40 min before the hexamethonium was given. Over this period there was an increase

in the vasoconstrictor response to noradrenaline similar to that following hexamethonium, and when this was included in an analysis of variance of the results the apparent effect of hexamethonium on the noradrenaline response was not significant.

It should be noted that, in these experiments, hexamethonium had no effect on the perfusion pressure of the hind limb whether innervated or not (Table 1).

DISCUSSION

Our experiments in the rat, like those in the cat reported by Zaimis (1956) and Mantegazza, Tyler & Zaimis (1958), demonstrate that the blood pressure response to repeated doses of hexamethonium becomes less with each dose. We have considered two possible explanations of this effect.

First, a decrease in the depth of anaesthesia during the experiment might allow an increase in cardiovascular reflex activity. This has been shown to be unlikely. The duration of anaesthesia had no influence on the initial blood pressure response to hexamethonium or on the lessening of the response to subsequent doses.

A second explanation has been suggested by Zaimis and her colleagues (Zaimis, 1955, 1956; Mantegazza, Tyler & Zaimis, 1958) on the basis of studies in the cat, namely, that hexamethonium by a local action can sensitize blood vessels to the constrictor effect of noradrenaline sufficiently to mask the effect of its ganglion-blocking action. We have attempted to obtain evidence of this in the rat by perfusing one hind limb at constant rate with the rat's own blood. Because the perfusion was at constant rate, the perfusion pressure should depend only on the combined vasoconstrictor nervous activity and vascular reactivity. Hexamethonium will reduce vasoconstrictor activity by ganglion blockade. It may also enhance vascular reactivity by a local action. If such local action is sufficient to overcome the effects of the ganglion blockade as postulated, the falls in hind-limb perfusion pressure produced by successive doses of hexamethonium should follow the blood pressure responses and become smaller. We did not find this in the rat. With successive doses of hexamethonium the hind-limb perfusion pressure fell by the same proportion each time. Nevertheless, the blood pressure response became less with each dose. This result shows that, at least in the hind limb, hexamethonium does not sensitize blood vessels to noradrenaline enough to mask the effect of its ganglion-blocking action. It is not of course denied that hexamethonium may have a slight local sensitizing action on blood vessels.

In the experiments with larger doses of hexamethonium, the increasing failure of the hind-limb perfusion pressure to recover after each dose no doubt reflects the increasing ganglion blockade resulting from the cumulation of hexamethonium in the circulation. In spite of this evident cumulation, the blood pressure was able to return to normal after each dose of hexamethonium. This is in accord with the observations of Zaimis (1956) in the cat. She found that the response of the nictitating membrane to preganglionic stimulation was increasingly reduced by successive doses of hexamethonium while the blood pressure response became less.

It is possible that changes in pressure at the venous outflow of the hind limb or in any collateral circulation in the hind limb occurred in our experiments so that

the hind-limb perfusion pressure was not simply dependent on the vasoconstrictor nervous activity and vascular reactivity as we have assumed. However, when the rat was prepared so that any collateral circulation was removed, the results were the same as in the simpler preparation. Even when the hind limb was completely isolated except for its nervous connexions and perfused with blood from a donor rat, the results were essentially similar. In this case there was no hexamethonium circulating in the hind limb. It would appear then that any pressure changes at the venous outflow or in any collateral circulation in the hind limb are not important.

Our experiments with the isolated hind-limb preparation suggest that any effect of hexamethonium on the response of the hind-limb blood vessels to noradrenaline is small. Statistically significant effects were only observed when the innervated hind-limb preparations were used without adequate control experiments. The fact that the level of perfusion pressure did not increase when hexamethonium was added to the blood perfusing the innervated hind limb supports the conclusions that any local action of hexamethonium is small. Any large local action would be expected to enhance the effect of the noradrenaline released endogenously by the vasoconstrictor nerves.

From our results we cannot say why the blood pressure response to repeated doses of hexamethonium becomes less with each dose in the anaesthetized rat. It is clear that local effects of hexamethonium on the blood vessels of the hind limb are not sufficient to mask the ganglion-blocking effects. Evidently the changes which reduce the blood pressure response occur elsewhere in the cardiovascular system, presumably in the heart or possibly in blood vessels other than in the hind limb.

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A NOTE ON THE DISTRIBUTION OF [^{14}C]-HISTAMINE ADDED TO BLOOD

BY

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(*Received May* 16, 1961)

^{14}C-Labelled histamine was incubated with canine or human blood at 37° C. After 1 hr 80 to 100% of the added histamine could be recovered in unchanged form. When [^{14}C]-histamine was added to whole blood *in vitro* it tended to become equally distributed between the cell and plasma fraction of the blood, and when the cell fraction of blood containing [^{14}C]-histamine was suspended in [^{14}C]-histamine-free plasma the labelled histamine tended to become equally distributed between cells and plasma. The pattern of distribution of intravenously injected [^{14}C]-histamine in the blood of anaesthetized dogs seemed to be the same as that of histamine added *in vitro*. The injected histamine entered the cell fraction of the blood at a slow rate. These experiments indicate that in dog and man the blood cells are of little or no importance for the inactivation of histamine released into the circulating blood.

Anrep & Barsoum (1935) and Anrep, Barsoum, Talaat & Wieninger (1939) studied the distribution of histamine added to blood. They found that both red and white blood cells easily took up considerable quantities of histamine added *in vitro*, but only when the histamine concentration of the plasma was higher than that of the cells. They also reported that the histamine taken up by the cells was not, or only to a small extent, released again when the cells were resuspended in histamine-free plasma. Since the quantities of histamine used were rather large, this might mean that the blood cells could take part in the inactivation of histamine reaching the blood in the living organism. Similar experiments have now been done with ^{14}C-labelled histamine, assayed by the isotope dilution technique developed by Schayer (Schayer & Cooper, 1956). This made it possible to assay for the added histamine in the presence of histamine originally present in the blood. It was possible to work with smaller more " physiological " quantities of histamine (0.03 to 0.05 μg histamine base per ml. blood). Three types of experiments were carried out: (1) Studies of the inactivation of [^{14}C]-histamine added to canine or human blood *in vitro*. (2) Studies of the distribution of [^{14}C]-histamine added to canine or human blood *in vitro*. (3) Studies of the distribution in the blood of [^{14}C]-histamine injected intravenously into dogs.

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METHODS

Experiments on blood incubated in vitro. Blood from 5 dogs and one man was used. Canine blood was taken with a polythene catheter inserted into a femoral artery under local anaesthesia. Human blood from a cubital vein was obtained with an internally polished cannula of stainless steel. The blood was collected in polythene tubes containing 0.15 ml. of a heparin solution (5 mg/ml.) per ml. blood. After adding histamine labelled with ^{14}C in the 2 position of the imidazole ring (specific activity 4.75 mC per mM; Radiochemical Centre, Amersham, England), the sample was well mixed and then placed in a shaking incubator at 37° C for varying periods of time. To separate cells from plasma the blood was centrifuged for 10 min at a rate of 10,000 rev/min in a refrigerated centrifuge (the diameter of the centrifuge head was about 20 cm). Samples of the blood were taken with silicone-treated pipettes, and the isotope dilution assays were carried out as described by Lindell & Schayer (1958).

Distribution of histamine in vivo. Two dogs (one male and one female) weighing 9 to 12 kg were fasted overnight and anaesthetized with sodium pentobarbitone (30 mg/kg) intravenously.

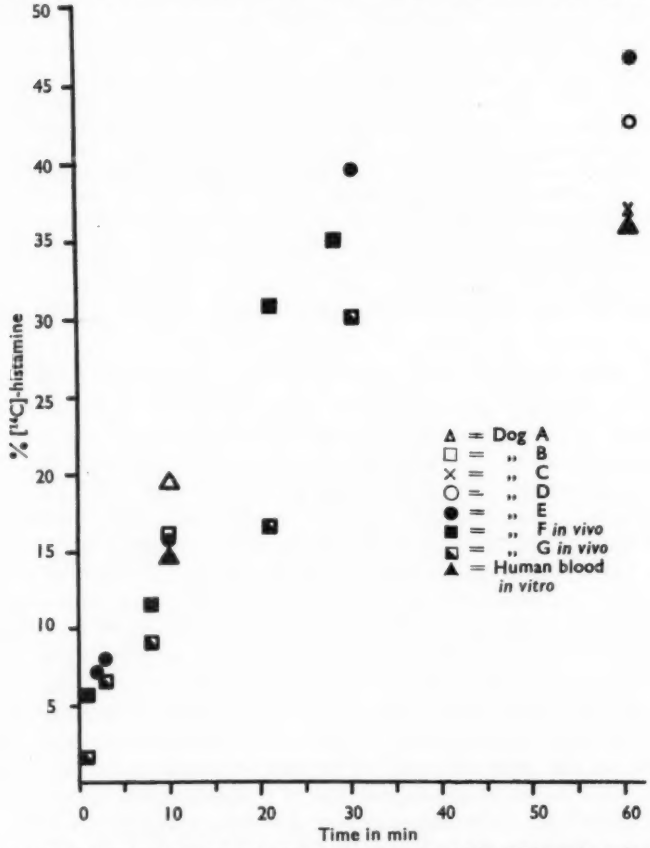

Fig. 1. The distribution between cell and plasma fraction of [^{14}C]-histamine added to human and canine blood. Abscissa: Duration of incubation of [^{14}C]-histamine with whole blood (shaking incubator at 37° C). Ordinate: The amount of [^{14}C]-histamine found in cell fraction (expressed as % of [^{14}C]-histamine content of whole blood).

[^{14}C]-Histamine dissolved in saline was infused with a motor-driven syringe into a catheter tied into a branch of the left femoral vein, at a rate of 0.6 to 1.2 μg/kg/min histamine base. Arterial blood was collected from a polythene catheter in the right femoral artery and transferred to the centrifuge as quickly as possible.

RESULTS

When [^{14}C]-histamine had been incubated with canine or human blood for 1 hr at 37° C, 80 to 100% of the added histamine could be recovered in unchanged form. Since the quantity of labelled histamine was 0.03 to 0.05 μg/ml. blood, this would mean that 0.01 μg histamine or less was metabolized in 1 hr by 1 ml. blood.

Fig. 1 shows the distribution of [^{14}C]-histamine added to whole blood. It may be seen that the amount of [^{14}C]-histamine in the cell fraction of the blood increased with increasing time of incubation. After 5 min of incubation about 5% of the labelled histamine was in the cell fraction, and after 1 hr 35 to 47% of the added histamine was found in the cell fraction of the blood. The distribution of added histamine seemed to be the same in human blood as in canine blood. The distribution of injected [^{14}C]-histamine in the blood of two dogs is also shown in the figure.

In the next series (Fig. 2) [^{14}C]-histamine was incubated with whole blood for 1 hr. The blood was then centrifuged and the cell fraction suspended in [^{14}C]-

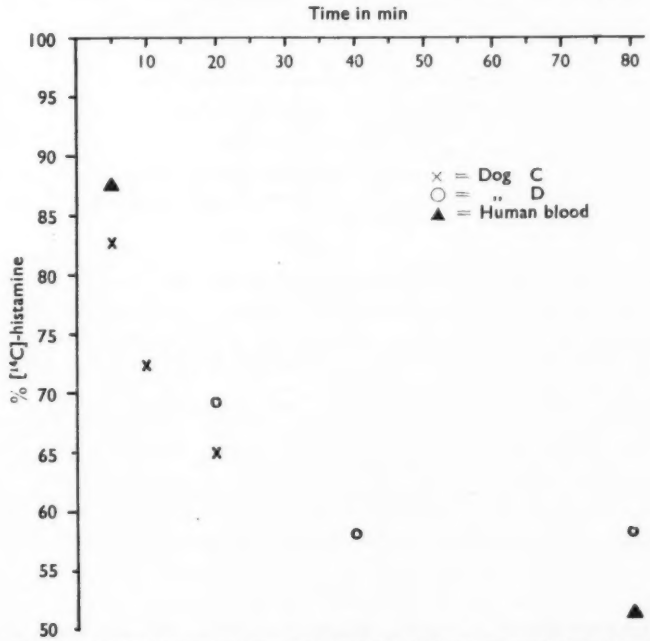

Fig. 2. Release of [^{14}C]-histamine from cell fraction of blood suspended in [^{14}C]-histamine-free plasma. Abscissa: Duration of incubation of [^{14}C]-histamine containing cell fraction in [^{14}C]-histamine-free plasma (shaking incubator at 37° C). Ordinate: The amount of [^{14}C]-histamine found in cell fraction (expressed as % of [^{14}C]-histamine content of whole blood).

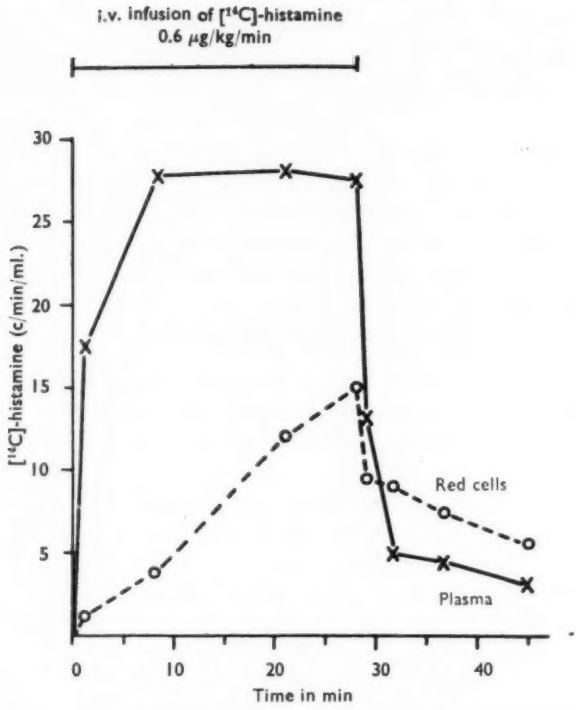

Fig. 3. Distribution of intravenously injected [^{14}C]-histamine between plasma and cell fraction of arterial blood from a male dog weighing 9 kg. Abscissa: Duration of experiment. Ordinate: [^{14}C]-Histamine content of cell and plasma fraction.

histamine-free plasma. After thorough mixing this blood was placed in the shaking incubator and samples removed for isotope dilution assay at various intervals. After 5 min of resuspension in histamine-free plasma the cell fraction contained 80 to 90% of the labelled histamine, and after 80 min 50 to 60% of the radioactive histamine was in the cell fraction.

Figs. 3 and 4 show the results of two experiments *in vivo*. During the intravenous infusion of [^{14}C]-histamine the concentration of labelled histamine in the blood plasma rose rapidly, but that of the cell fraction rose much more slowly. When the infusion was stopped the concentration of [^{14}C]-histamine in the plasma fell very rapidly; that in the cell fraction of the blood also dropped, but more slowly.

DISCUSSION

It was found that of [^{14}C]-histamine added to canine or human blood *in vitro* very little, if any, was metabolized. There was no evidence of any significant enzymic inactivation of histamine in heparinized whole blood. It should be mentioned that Blaschko, Friedman, Hawes & Nilsson (1959) found that dialysed

dog serum sometimes oxidized histamine. Later the same year Blaschko expressed doubts about the physiological significance of the serum enzymes for the inactivation of histamine.

[^{14}C]-Histamine added to whole blood tended to become equally distributed between the cell and the plasma fractions. This result is similar to that of Anrep & Barsoum (1935) and Anrep *et al.* (1939), who worked with non-labelled histamine in much higher concentrations.

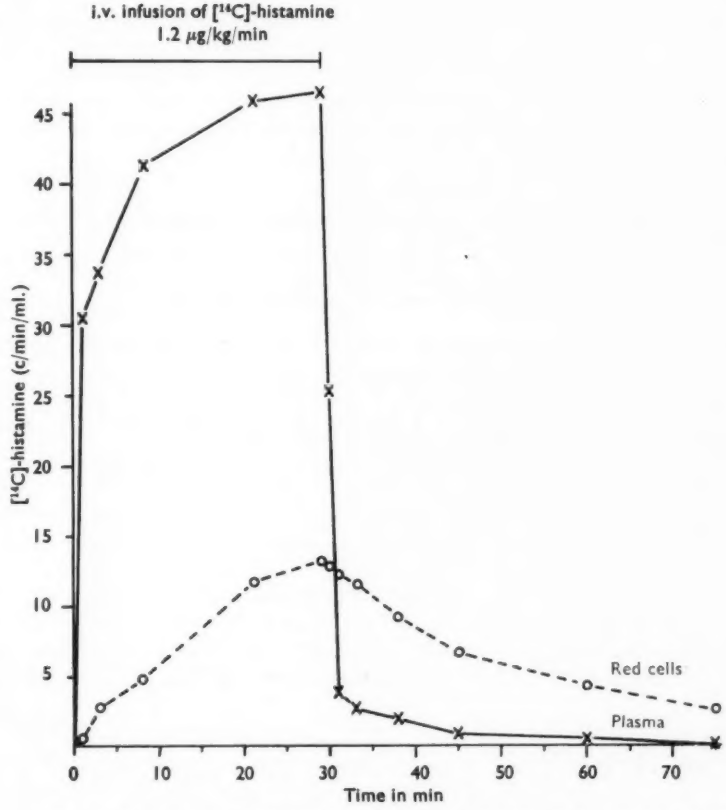

Fig. 4. Female dog weighing 12 kg. Conditions otherwise as described in Fig. 3.

When the cell fraction from blood containing [^{14}C]-histamine was resuspended in [^{14}C]-histamine-free plasma, radioactive histamine moved into the plasma and tended to become equally distributed between the cell and plasma fraction of the blood. This is not in agreement with the results of Anrep & Barsoum and their colleagues. It might be argued that in the present experiments the cell fraction released histamine into the plasma as a consequence of cell damage. However, the results of the experiments *in vivo* do not support such an assumption.

It would be reasonable to assume that some exchange of added histamine took place between plasma and cell fraction during the centrifugation procedure. However, only 5% of the histamine added to whole blood was found in the cell fraction after incubation for 5 min and centrifugation for 10 min. This indicates that any exchange of labelled histamine between cells and plasma during the centrifugation was small.

The infusion experiments indicate that the uptake of histamine by blood cells is too slow to be of significance in the inactivation of histamine that has been released into the blood. The cell fraction released [^{14}C]-histamine which it had taken up only when the [^{14}C]-histamine concentration in the plasma fell below that of the cell fraction. These observations are in agreement with the finding by Schayer & Cooper (1956) and by Nilsson, Lindell, Schayer & Westling (1959) that after the injection of ^{14}C-labelled histamine in man the radioactivity is rapidly excreted in the urine.

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SENSITIVITY OF THE HYPERTHYROID AND HYPOTHYROID MOUSE TO HISTAMINE AND 5-HYDROXYTRYPTAMINE

BY

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Injections of thyroxine sodium increased the sensitivity of mice to histamine, the maximal effect occurring after 6 days. The sensitivity to 5-hydroxytryptamine was increased to a lesser extent. Hydrocortisone antagonized this effect of thyroxine, whereas anti-thyroid drugs alone decreased the sensitivity to histamine. Treatment with thyroxine also raised the metabolic rate, the peak again being reached after 6 days. As treatment was continued beyond 6 days, the adrenal gland enlarged and its secretion probably accounted for the decline in the histamine sensitivity.

During a study of the influence of the endocrine glands on the sensitivity of rats to an intraperitoneal injection of egg-white, Léger & Masson (1948) noted that injections of thyroid extracts produce a striking modification of the anaphylactoid reaction. This reaction in untreated rats is characterized by gross oedema of the extremities with recovery within 6 hr, but a shock-like condition is produced within a few minutes of the egg-white injection into thyroxine-treated rats and many of the animals die. Parratt & West (1960) confirmed this result, and showed that the cause of death is internal oedema and haemorrhage in the intestinal tract. These latter authors also found that after thyroxine treatment the histamine released by the injection of egg-white accumulates in the blood, possibly as a result of inhibition of histaminase, an enzyme responsible for its inactivation. Parratt & West (1957) had previously reported that egg-white releases both histamine and 5-hydroxytryptamine in the rat and that both amines play roles in producing the anaphylactoid reaction.

The mouse, like the rat, is resistant to the systemic effects of histamine and 5-hydroxytryptamine, but this resistance may be lowered by pre-treatment with *Haemophilus pertussis* vaccine (Parfentjev & Goodline, 1948; Kallós & Kallós-Deffner, 1957) or by adrenalectomy (Halpern & Wood, 1950; Munoz, 1957). It was of interest, therefore, to determine whether mice are rendered supersensitive to histamine and 5-hydroxytryptamine after treatment with thyroid hormones, and whether anti-thyroid drugs have the opposite effect.

METHODS

Groups of at least 10 male mice (initial body weight 20 to 25 g) were used in each experiment. They were housed at 72±1° F and fed on a cube diet (Associated Flour Millers, No. 41B). Drinking water was allowed *ad libitum*.

Drug treatment. Animals were injected daily with suitable dilutions of a stock solution of L-thyroxine sodium (2.5 mg/ml.) in 0.9% sodium chloride solution containing 0.01N sodium hydroxide. The dilutions were prepared daily so that the volumes injected subcutaneously were constant at 0.1 ml. The doses of thyroxine sodium used were 1, 2 and 5 mg/kg. Control animals received daily subcutaneous injections of the solvent.

In other experiments, daily intramuscular doses of hydrocortisone acetate (2 mg/kg) were given, alone and in combination with thyroxine sodium (2 mg/kg). To produce hypothyroidism, methylthiouracil (0.2% w/w) was added to the diet for 10 days, or methimazole (0.2% w/v) or carbimazole (0.15% w/v) was included in the drinking water for 10 days.

Sensitivity to histamine and 5-hydroxytryptamine. 24 hr after the last injection of thyroxine, the sensitivity to a standard intraperitoneal dose of histamine (40 mg/kg) or 5-hydroxytryptamine (20 mg/kg) was tested in each mouse using an arbitrary shock-score system, as follows: Sedation, cyanosis and defaecation, recovery within 60 min, score of 1; profound sedation and unsteady gait, recovery within 90 min, 2; severe shock and respiratory distress, recovery within 24 hr, 3; death within 24 hr, 5; death within 5 hr, 6; death within 1 hr, 7; death within 40 min, 8; death within 20 min, 10.

On this basis, an animal possessing the maximal degree of sensitivity (that is, a score of 10) convulses and dies within 20 min of the challenge injection. The maximal score of each group of 10 mice is therefore 100, and the sum of the scores in each group gives an estimate of the degree of sensitivity to each drug. The average score of a group of 10 untreated mice is 10, and values in excess of 20 are statistically different ($P=0.95$).

In other tests after treatment with thyroxine sodium or with the anti-thyroid drugs, the intraperitoneal toxicities of histamine and 5-hydroxytryptamine were determined. The LD50 values and their confidence limits were calculated by the method of Litchfield & Wilcoxon (1949), using at least 5 groups of animals for each determination. In the experiments with hydrocortisone, only the toxicity of histamine was estimated.

Resistance to anoxia. The survival times of mice subjected to anoxia were determined by placing the animals in individual airtight vessels of similar size, as used by Basil, Somers & Wollett (1950). The metabolic rates were then calculated as the volume of air used up/g mouse/min. A group of 10 untreated mice have a mean value (±s.e.) of 0.24±0.01 ml./g/min.

Hypertrophy of the adrenal gland. Both adrenal glands were removed from each mouse after treatment and weighed moist on a torsion balance. A group of 10 untreated mice have a mean value (±s.e.) of 4.25±0.25 mg.

RESULTS

Effect of thyroxine on histamine sensitivity. Treatment with thyroxine sodium raised the shock-score response of mice to a standard dose of histamine. This is shown in Fig. 1 for 3 different doses of thyroxine. With 1 mg/kg daily, the severity of the reaction to histamine increased after 4 days of treatment to reach a peak at 8 days; it then declined as thyroxine treatment continued and at 14 days it had almost returned to the initial level. With 2 and 5 mg/kg daily, the response started earlier, reached the peak sooner, and did not decline so fast as treatment continued.

The toxicity of histamine increased more than 10 times after treating the mice with thyroxine sodium (2 mg/kg). This is illustrated in Fig. 2, where the values after 6 and 14 days are plotted.

The profound increase in the toxicity of histamine produced by thyroxine, however, was prevented by the simultaneous administration of hydrocortisone, which

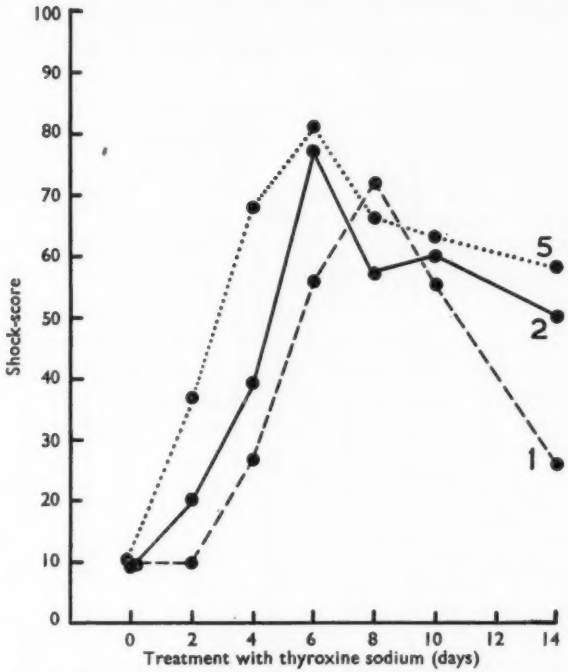

Fig. 1. The effect of treating mice with daily doses of thyroxine sodium on the sensitivity to histamine (40 mg/kg). Doses used were 1 (● - - - ●), 2 (●——●), and 5 (● · · · · ●) mg/kg. Sensitivity was assessed on a shock-score system. Ordinate: shock-score. Abscissa: time in days.

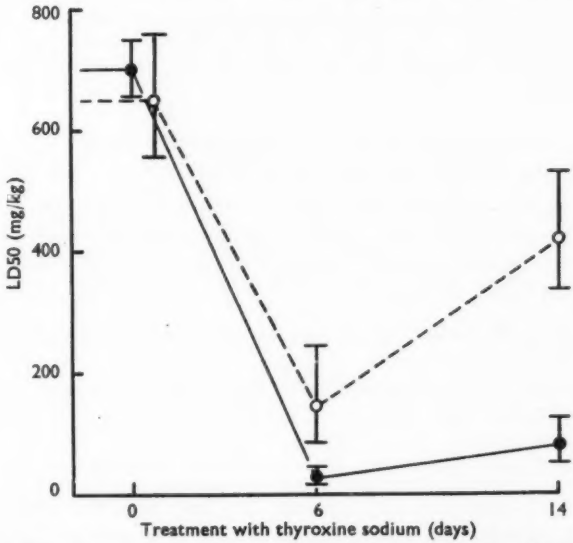

Fig. 2. The effect of daily doses of thyroxine sodium (2 mg/kg) on the toxicity of histamine (●——●) and 5-hydroxytryptamine (○- - -○) in mice. The LD50 values (mg/kg) and their confidence limits are recorded. Ordinate: LD50 (mg/kg). Abscissa: duration of treatment in days.

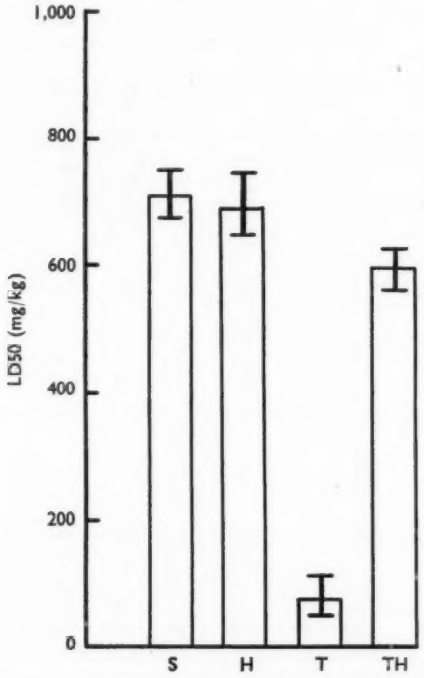

Fig. 3. The effect of 6 doses of thyroxine sodium (2 mg/kg), hydrocortisone acetate (2 mg/kg), and thyroxine and hydrocortisone together, on the toxicity of histamine in mice. S=solvent, H=hydrocortisone, T=thyroxine, and TH=thyroxine and hydrocortisone. Ordinate: LD50 in mg/kg.

by itself was without effect on the toxicity of histamine. The results of an experiment using 6 daily doses of thyroxine and hydrocortisone are shown in Fig. 3.

Effect of anti-thyroid drugs on histamine sensitivity. Treatment with each of the anti-thyroid drugs for 10 days significantly decreased the toxicity of histamine (Table 1). For example, methylthiouracil in the diet or methimazole in the drinking water raised the LD50 of histamine to over 1,000 mg/kg (control figure 725 mg/kg).

TABLE 1

THE EFFECT OF TREATING MICE WITH ANTI-THYROID DRUGS FOR 10 DAYS ON THE INTRAPERITONEAL TOXICITY OF HISTAMINE

	Histamine toxicity		
Treatment	LD50 (mg/kg)	Confidence limits	Level of signif cance
None	725	690– 761	—
Methylthiouracil	1,060	964–1,166	$P=0{\cdot}95$
Methimazole	1,020	953–1,091	$P=0{\cdot}95$
Carbimazole	930	816–1,060	$P=0{\cdot}95$

Effect of thyroxine on 5-hydroxytryptamine sensitivity. Treatment with thyroxine sodium failed to raise the shock-score response of mice to a standard dose of 5-hydroxytryptamine, irrespective of the dose of thyroxine used (1 to 5 mg/kg) or the number of doses (2 to 14). However, 6 doses of 2 mg/kg increased the toxicity of 5-hydroxytryptamine about 5-fold, although this increase was much reduced after 14 similar doses (Fig. 2). After anti-thyroid treatment (for example, methimazole in the drinking water for 10 days), the toxicity of 5-hydroxytryptamine was also unchanged.

Effect of thyroxine on metabolic rate. There was more than a 3-fold increase in the rate of exhaustion of air by mice after treatment with thyroxine sodium (2 mg/kg). The increase began after only 2 doses of thyroxine (Fig. 4), and the rate reached its peak after 6 days. This high rate was maintained for the next 8 days.

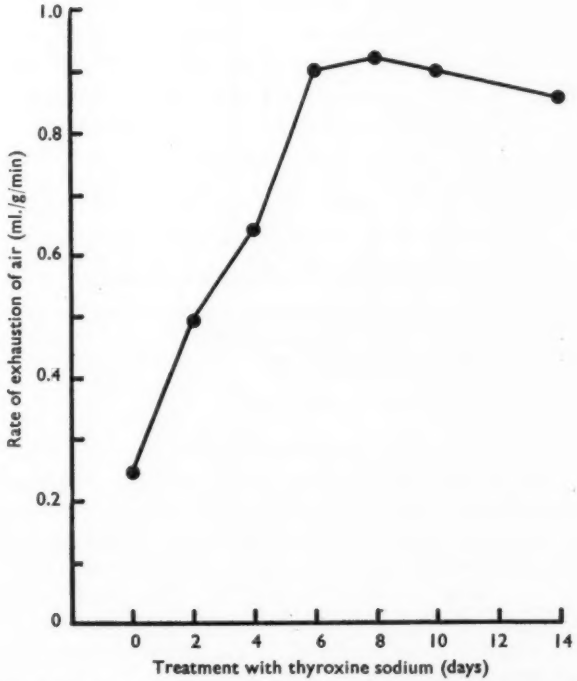

Fig. 4. The effect of daily doses of thyroxine sodium (2 mg/kg) on the metabolic rate of mice, as assessed by the anoxia method. Ordinate: rate of exhaustion of air is measured as ml./g/min. Each point is the mean value for a group of 10 mice. Abscissa: duration of treatment in days.

Effect of thyroxine on adrenal weight. Prolonged treatment of mice with thyroxine sodium (2 mg/kg) increased the adrenal weight, and after 14 days the glands weighed about twice the control value (Fig. 5).

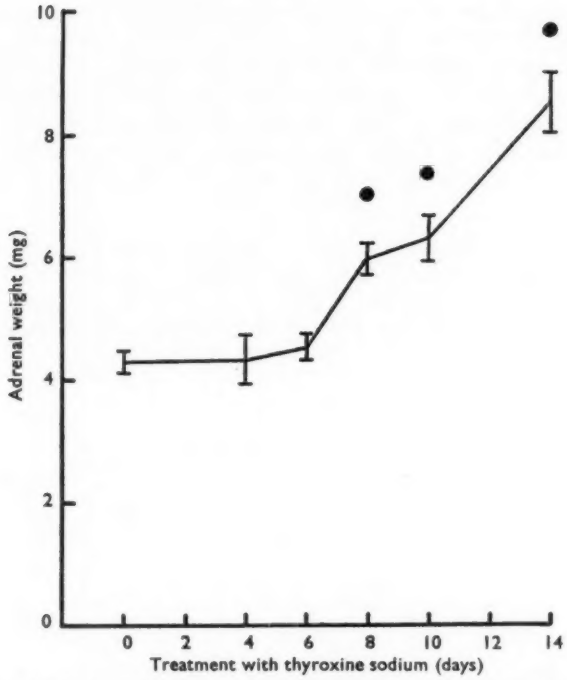

Fig. 5. The effect of daily doses of thyroxine sodium (2 mg/kg) on the combined weight (mg) of the adrenal glands of mice. Vertical lines represent standard errors of the means. The symbol ● denotes significant difference (P=0.95) from the control value. Ordinate: weight of adrenal glands in mg. Abscissa: duration of treatment in days.

DISCUSSION

The present results show that the mouse, like the rat, becomes very sensitive to injections of histamine when several doses of thyroxine sodium have been administered. This was shown when the response to a standard dose of histamine was measured and when the toxicity value of histamine was estimated. In both conditions, the maximum sensitivity occurred after 6 doses, after which there was a decrease. The changes in sensitivity took place simultaneously with changes in metabolic rate, when this was estimated by the anoxia method, and there may be a link between these two effects.

After prolonged treatment with thyroxine up to 14 doses, the mouse became less sensitive to histamine than it was after 6 doses, and this suggests that an internal compensatory mechanism has become effective. Examination of the adrenal glands indicated that after 8 doses of thyroxine there was gross enlargement, and this confirms the results of previous authors (Gardner, 1942; Maqsood, 1950). Deane & Greep (1947) have reported that the major changes within the adrenal glands take place in the cortex, the zona fasciculata in particular being stimulated. In the present

experiments, evidence has been obtained that hydrocortisone protected the hyperthyroid mouse against histamine, and it is probable that the animals' own glucocorticoids played a similar protective role after the prolonged treatment with thyroxine when the adrenal glands were enlarged and producing more of the cortical hormones.

The mechanism whereby sensitivity of the hyperthyroid mice to histamine was raised is not clear. Firstly, it may be due to an increased rate of absorption of injected histamine. The results of pilot experiments suggested that this is unlikely, since the increase in blood histamine of thyroxine-treated mice did not differ from that of untreated animals. Secondly, it may be the result of a decreased ability to inactivate histamine. Parratt & West (1960) showed that the histaminase activity of the rat ileum after thyroxine treatment is reduced and this results in the accumulation of histamine in the blood. However, blood histamine levels in the mouse continue to rise for 30 min, whereas many of the supersensitive animals died within 20 min. Anti-thyroid drugs exerted a protective action against histamine, and further work is indicated to determine their mode of action in this respect.

The changes in the sensitivity of the mice to 5-hydroxytryptamine in the present experiments have been minor. This amine became about 5 times more toxic to mice after thyroxine treatment, but this alteration in sensitivity did not show in the experiments using the shock-score system. The anti-thyroid drugs also failed to alter the sensitivity to 5-hydroxytryptamine. Further work is in progress to clarify this problem.

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ASSAY OF SUBSTANCE P ON THE FOWL RECTAL CAECUM

BY

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The optimum conditions for the assay of substance P on fowl rectal caecum have been studied. Effective concentrations vary from 0.01 to 0.5 u./ml.; it is remarkably insensitive to other polypeptides, such as bradykinin. The test can be made more specific by using a bath fluid containing antagonists for known interfering substances. A suitable antagonist for acetylcholine is atropine or hyoscine, for 5-hydroxytryptamine, methysergide and for catecholamines, ephedrine. The effects of histamine and adenosine compounds can be abolished by specific tachyphylaxis, in which the bath fluid contains an excess of the active substance itself. The assay is reasonably accurate. Woolf's index of precision (L) was estimated as 15.5 with a range (9) of 9.2 to 29.4. Simplified methods of calculating L, and the fiducial range, are described.

The effects of drugs on the muscle of the fowl rectal caecum were first studied by Barsoum & Gaddum (1935). This tissue has been used by Euler (1948) to discriminate between catecholamines, since it is about 40 times more sensitive to adrenaline than to noradrenaline. It is sensitive to the active polypeptide known as substance P, but remarkably insensitive to bradykinin and other kinins (Pernow & Rocha e Silva, 1955; Gomes, 1955). It can therefore be used to detect and estimate small amounts of substance P in the presence of these other polypeptides, but it is sensitive to many pharmacologically active substances which may be present in tissue extracts.

The effects of these other substances can sometimes be excluded by using suitable methods of extraction. Amin, Crawford & Gaddum (1954) used 95% acetone to extract acetylcholine, catecholamines, 5-hydroxytryptamine and histamine, leaving substance P in the insoluble residue, together with adenosine compounds and possibly other substances. It is, however, difficult to be sure that such methods will always be effective. Amin *et al.* concluded that two extractions with acetone were sufficient, since no 5-hydroxytryptamine was detected in the third extract. We have made similar extracts and compared the acetone-insoluble fraction with substance P, using the guinea-pig ileum; when the experiment was repeated after the ileum had been desensitized to 5-hydroxytryptamine by adding tryptamine to the bath, the substance P equivalent of the extract was decreased. This result suggests that some of the 5-hydroxytryptamine had not been removed by the acetone, either because it was present in a combined form or for some other reason. Other evidence of this is described below.

An alternative method of eliminating the effects of other pharmacologically active substances is to add suitable antagonists to the bath fluid, so as to make the test more specific. The experiments described below were undertaken to find the best conditions for using the rectal caecum as a specific test for substance P.

METHODS

Fowl rectal caecum. The narrow part of the rectal caecum was removed from the hen and stored in modified Tyrode solution at 4° C for 2 to 40 hr. The optimum duration of storage was about 18 hr; after storage the muscle had less spontaneous activity but was sometimes less sensitive to drugs. A length of about 4 to 5 cm was suspended in a bath (3 to 5 ml.). The movements of the longitudinal muscle were recorded on a smoked drum, with an amplification of 15 and a tension of 1 to 5 *g*.

The bath fluid was that used by Barsoum & Gaddum (1935). Its composition is as follows: sodium chloride 8, potassium chloride 0.2, calcium chloride 0.2, magnesium chloride 0.01, sodium bicarbonate 1, sodium dihydrogen phosphate 0.05, glucose 1 g/l. It is identical with Tyrode solution, but contains only 10% of the usual amount of magnesium chloride.

In some experiments, in order to eliminate the effect of the accumulation of active substances in the small bath, a slow flow of fluid was allowed to pass through the bath from below, between the additions of drugs, and sucked out from above (Schild, 1947; Fastier & Reid, 1949). This flow was stopped 15 sec before the drugs were added and the drugs were usually left in contact with the tissue for 30 sec. The fluid was then allowed to run in quickly from below until the bath contents were changed. The whole cycle usually occupied 4 min.

In the first experiments, air was bubbled through the bath, but when this was replaced by 95% oxygen with 5% carbon dioxide the spontaneous contractions decreased and the responses to substance P and acetylcholine increased. This mixture of gases was therefore used in the later experiments.

Estimation of purine (adenine and guanine) compounds. The extracts (20 mg acetone powder/ml.) were treated with perchloric acid to give a final concentration of 5% and allowed to stand for at least 1 hr at 4° C. After centrifuging the mixture, the supernatant was decanted and neutralized with 3N potassium hydroxide. The mixture was centrifuged again, the supernatant was diluted 10 times and used for determination of specific absorption at 260 mμ (Kalckar, 1947). Extracts prepared from intestine had a specific absorption equivalent to 16 nM/mg or 5.3 μg/mg acetone powder (calculated as adenosine monophosphate). Extracts prepared from brain usually contained the equivalent of 18 nM/mg or 6 μg/mg acetone-dried power. These figures are comparable with those of other workers (McIlwain, 1959).

On a few occasions the adenosine triphosphate was measured with the firefly luminescence method (Strehler & Totter, 1954), and amounts varying from 10% to 25% of the total purine were found.

Tissue extracts. Acetone-dried powders of fresh tissue were prepared by three extractions with 20 vol. of dry acetone. In some experiments the powders were then ground in a mortar with 2 vol. *n*-butanol and the mixture heated at 75° C for 30 min. The butanol was removed by evaporation at 60° C under reduced pressure. This treatment with butanol was found by Leach (1959) to increase the activity by 35%, but the explanation of this phenomenon is still unknown. The dry powder was extracted with 0.02 M acetate (or citrate) buffer *p*H 5.5 containing 0.9% (w/v) sodium chloride, by heating for 10 min in a boiling water bath. The extract was either used the same day or dried for storage. The buffer solution alone had no effect on the caecum.

Drugs used. In many of the experiments the preparation of substance P was one made by Hoffmann La Roche & Co. from horse intestine by the methods used by Pernow (1953). It contained 75 Euler u./mg, and has been used as a standard preparation in many laboratories. In other experiments a laboratory standard was used. This was prepared from cattle brain using similar methods. A solution of the ammonium sulphate precipitate was passed through

one alumina column and freeze-dried. It was compared with the first preparation on guinea-pig ileum and estimated to contain 12 u./mg. It was also tested on rat uterus, fowl rectal caecum and rabbit jejunum, and similar results were obtained. It lost more than 90% of activity on incubation with chymotrypsin. These results suggest that the two preparations owe their activity to the same substance.

A preparation of synthetic bradykinin was kindly supplied by Parke, Davis & Co. Preparations of lysergic acid diethylamide, 2-bromolysergic acid diethylamide, 1-methyl-lysergic acid butanolamide (methysergide) and dihydroergotamine were presented by Sandoz & Co.

Phenoxybenzamine (Dibenzyline) and dibenamine (*NN*-dibenzyl-β-chloroethylamine) were presented by Smith, Kline & French Co. Triprolidine (295C51 ; trans-1-(4′-methylphenyl)-1-(2′-pyridyl)-3-pyrrolidinoprop-1-ene) was presented by Dr A. F. Green.

RESULTS

Sensitive preparations often had much spontaneous activity, which obscured the record. Attempts have therefore been made to reduce spontaneous movements without reducing sensitivity ; storage at 4° C or the addition of ephedrine or adenosine monophosphate had some effect (see below).

Inorganic salts. Some of the effects of these were described by Barsoum & Gaddum (1935). A few experiments were undertaken in the hope of finding a more suitable bath fluid. When the concentration of magnesium chloride was increased to that present in Tyrode solution (0.1 g/l.) the spontaneous movements decreased, but the response to drugs also decreased.

Reduction of the concentration of potassium chloride to one-half had the same two effects. These changes in inorganic salts conferred no apparent benefit.

Temperature. The optimum temperature appeared to be 34 to 35° C. At 30° C the spontaneous movements were less, but so also was the response to substance P. Increasing the temperature to 38° C sometimes increased the response to substance P, but also increased spontaneous movements and the effect of adrenaline.

Age and sex of fowl. Preparations made from chickens 10 days old responded to substance P, but the results were not satisfactory when the age was less than 9 weeks ; the optimum age seemed to be 14 to 20 weeks. Cocks and hens were equally satisfactory.

Substance P. The concentration of substance P which caused a reasonable contraction of the fowl rectal caecum was 0.1 to 0.5 u./ml. and was not significantly different from the concentrations causing a similar contraction of isolated preparations of rabbit jejunum, guinea-pig ileum, guinea-pig rectum or rat uterus. However, in the presence of adenine nucleotides the caecum often responded to 0.01 u./ml. (see below). The fowl rectal caecum is relatively insensitive to bradykinin, which causes a slower contraction and is difficult to compare accurately with substance P (Pernow & Rocha e Silva, 1955). It was estimated that 1 u. of substance P was equivalent to more than 10 μg of bradykinin.

The rat uterus, on the other hand, is about 1,000 times more sensitive to bradykinin and can be used to detect small amounts of bradykinin present as an impurity in preparations of substance P. The guinea-pig ileum is intermediate between these two preparations, being more sensitive to bradykinin than the fowl rectal caecum and less sensitive than the rat uterus (Gaddum, 1955).

The most important known substances likely to be present in tissue extracts are given in the following list, together with the concentrations (μg/l.) found to cause a small contraction of the rectal caecum: acetylcholine 5; histamine 10 or more; 5-hydroxytryptamine 100 to 1,000; bradykinin 10,000. Adrenaline (1), noradrenaline (40), adenosine (100), adenosine monophosphate (200) or adenosine triphosphate (400) caused inhibition.

Antagonists

Atropine (10^{-7}) decreased the sensitivity to acetylcholine by 200 times. Hyoscine is effective in the same concentration and was used in many of the tests.

Antagonists of catecholamines

Ephedrine. A concentration of $1–5 \times 10^{-6}$ in the reservoir usually relaxed the muscle and inhibited spontaneous movements. The effects of histamine, substance P and 5-hydroxytryptamine were often increased, and the inhibitory effect of adrenaline or noradrenaline was decreased.

In Fig. 1 it will be seen that the effect of 0.2 u. substance P was decreased when it was mixed with 1 ng of adrenaline. The addition of ephedrine (10^{-6}) to the bath fluid inhibited the tone of the muscle and the spontaneous movements.

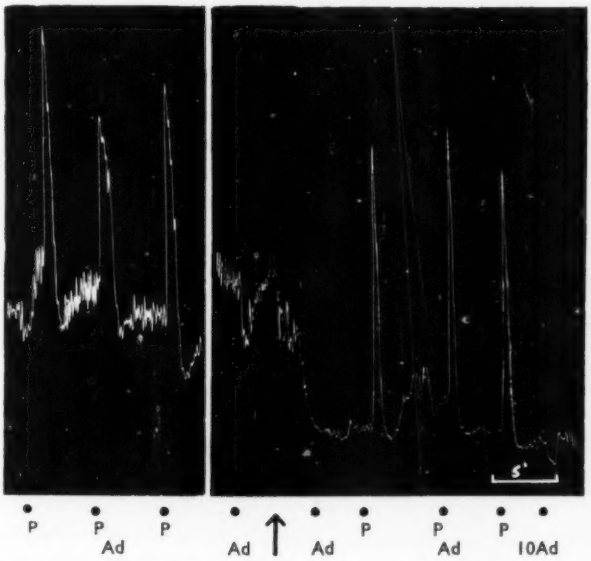

Fig. 1. Fowl rectal caecum suspended in a 5 ml. bath containing Tyrode solution (prepared with 10% of the usual amount of magnesium chloride). Drugs were added at 4 min intervals and allowed to act for 30 sec. P=substance P (0.2 u.); Ad=adrenaline (1 ng). From the arrow (↑) until the end of the tracing ephedrine (10^{-6}) was present in the bath.

Adrenaline now had no inhibitory effect by itself and did not diminish the response to substance P when the two substances were mixed. Higher concentrations of ephedrine (10^{-5}) eventually made the preparation less sensitive to substance P. Lower concentrations ($<10^{-6}$) were less effective in diminishing the response to adrenaline.

The noradrenaline content of extracts of parts of the dog brain was estimated by Vogt (1954), and her figures were compared with estimates of substance P by Amin *et al.* (1954). It will be seen from their Table 6 that in different tissues 1 u. substance P was associated with 0.25 to 25 ng noradrenaline, or approximately 1/10th of this quantity of adrenaline. The ratio for the intestine probably lies within this range. In these tissues the amount of adrenaline is thus likely to be less than 2.5 ng/u. substance P, which is slightly less than the amount which interferes with assays on the fowl rectal caecum (Fig. 1). Noradrenaline is less likely to interfere, though its concentration in the brain is about 10 times that of adrenaline, since its activity on the rectal caecum is about 1/40. Extraction with acetone removes most of the catecholamines which are then even less likely to interfere with the assay.

The presence of ephedrine in the bath is thus perhaps unnecessary for many purposes, but it does have some antiadrenaline action and appeared to be beneficial in other ways; it was, therefore, often present in the bath in experiments with other drugs.

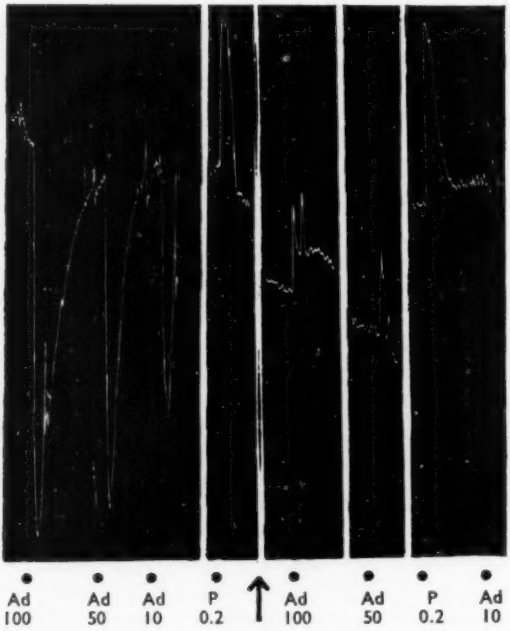

Fig. 2. As Fig. 1. Doses of adrenaline in ng and of substance P in u. From the arrow (↑) until the end of the tracing dichloroisoproterenol (10^{-6}) was present in the bath.

Dichloroisoproterenol (1-(3'4'dichlorophenyl)-2-isopropylaminoethanol). This substance antagonizes many of the inhibitory effects of adrenaline (Powell & Slater, 1958). It is thought to act on β-receptors (Ahlquist & Levy, 1959). Fig. 2 shows that it blocks the inhibitory effect of adrenaline on the rectal caecum. The muscle was inhibited by doses of 5 to 100 ng of adrenaline. When dichloroisoproterenol (10^{-6}) was added to the bath, this effect was reversed. Large doses of adrenaline now caused a brief contraction of the muscle, while small doses had practically no effect. In other experiments, similar to that shown in Fig. 1, substance P was mixed with adrenaline. Initially, this reduced the response, but when dichloroisoproterenol (10^{-6}) was added to the bath it increased the response. This reversal of the effect of adrenaline on the response to substance P occurred when the dose of adrenaline was only 10 ng. This drug is therefore not an ideal routine antagonist for adrenaline, but could be used to test for the presence of adrenaline in extracts.

Mushroom extracts. Mushroom extracts were used by Garven (1956) to inactivate adrenaline in the assay of 5-hydroxytryptamine. They have been found to destroy substance P.

Antagonists of histamine

Phenoxybenzamine (N-benzyl-N-phenoxyisopropyl-β-chloroethylamine). This substance irreversibly blocks the action of various drugs. It blocks the motor actions of adrenaline, but does not block the inhibitor actions (Nickerson, 1949). It was therefore not surprising that it did not antagonize the inhibitor action of adrenaline on the rectal caecum. Phenoxybenzamine has been found to be a particularly powerful antagonist of histamine on the rectal caecum. Fig. 3 shows an experiment in which 0.05 μg histamine in the presence of ephedrine caused an effect similar to that of

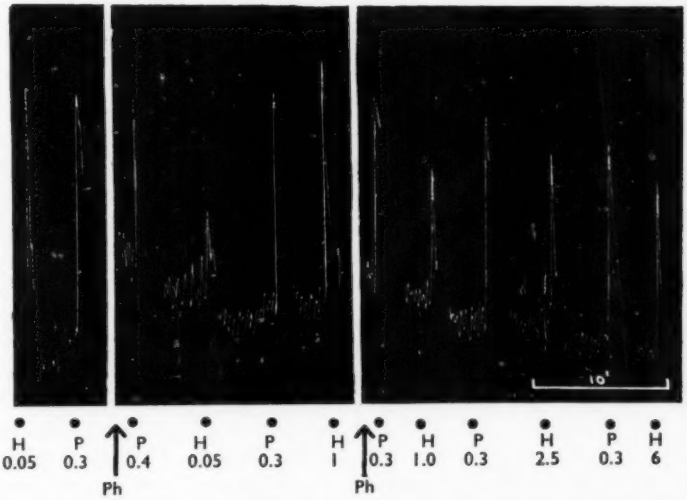

Fig. 3. As Fig. 1. Doses of substance P in u. and of histamine in μg. At the arrows phenoxybenzamine (10 μg) was added to the bath for 10 min.

0.3 u. substance P. A dose of 10 μg phenoxybenzamine was then added to the bath for 10 min and washed out again. This had no effect on the response to substance P, but decreased the response to histamine. The treatment with phenoxybenzamine was then repeated, and the effect of histamine was reduced still further, so that 6 μg now had less effect than 0.3 u. substance P. The relative potency of histamine had thus been reduced more than 120 times.

Other histamine antagonists. Dibenamine had a comparatively small effect on the action of histamine and reduced that of substance P. Mepyramine (10^{-6}) or promethazine (10^{-7}) reduced the effect of histamine, but did not abolish it. Higher concentrations reduced the effects of both histamine and substance P. Triprolidine (Green, 1953) is a particularly active and specific antihistamine on other tissues. A concentration of 10^{-6} (in the presence of ephedrine) reduced the sensitivity to histamine 3 times without altering the response to substance P. A concentration of 10^{-5} reduced the sensitivity to histamine 30 times, but reduced also the response to substance P.

A high concentration of histamine itself in the bath was found by Barsoum & Gaddum (1935) to cause a contraction lasting only a few min, in spite of the continued presence of the drug in the bath. This observation has been confirmed; a concentration of 0.5 to 2×10^{-6} of histamine reduced the sensitivity to histamine by 50 to 200 times. The response to substance P was in some cases unaffected, but in others it was reduced. In many of the experiments on extracts histamine (2×10^{-6}) was present in the bath.

Antagonists of 5-hydroxytryptamine

Many preparations were rather insensitive to 5-hydroxytryptamine, but a concentration in the bath of 0.1 to 2×10^{-6} often produced a biphasic contraction. An excess of tryptamine in the bath has been found to desensitize some plain muscle to both the tryptamine itself and to 5-hydroxytryptamine (Gaddum, 1953a); but this was not effective with fowl rectal caecum; a concentration of 5×10^{-6} caused a prolonged contraction with little or no tachyphylaxis.

Phenoxybenzamine, in a concentration of 2×10^{-6} for 10 min, had no effect, but double this concentration for the same time made the muscle 10 times less sensitive to 5-hydroxytryptamine.

Various derivatives of lysergic acid acted as specific antagonists of 5-hydroxytryptamine when added to the bathing fluid, so as to be indefinitely in contact with the muscle; lysergic acid diethylamide, in a concentration of 10^{-7}, made the muscle 3 to 10 times less sensitive. The 2 bromo-derivative and dihydroergotamine had a similar effect. The most satisfactory results were obtained with methysergide. In a concentration of 5×10^{-8} this reduced the sensitivity of the muscle to 5-hydroxytryptamine by more than 100 times and had little or no effect on the response to substance P. According to Amin *et al.* (1954), extracts of horse intestine contain less than 20 ng of 5-hydroxytryptamine/u. substance P, and extracts of various parts of dog brain all contained less than this. A dose of 0.3 u. of substance P is thus likely to be mixed in an extract with not more than 6 ng of 5-hydroxytryptamine,

which would give a concentration in the bath of less than 2×10^{-9}, and this would have no effect, even in the absence of antagonists. An example is given below, however, of an experiment where 5-hydroxytryptamine was present in just sufficient concentration to affect the result when no antagonist was used. It is thus generally not necessary to use an antagonist for 5-hydroxytryptamine in experiments with the rectal caecum, but it is advisable to check the sensitivity to 5-hydroxytryptamine on each occasion.

Purine compounds

Contractions of the fowl rectal caecum are inhibited by low concentrations of adenosine compounds. The most active is adenosine itself, which has an effect in a concentration of 10^{-7} as was first observed by Barsoum & Gaddum (1935). We have found that when this same concentration was mixed with substance P (about 0.25 μg of adenosine/u. substance P) it caused a small diminution of the response; smaller doses of adenosine had no effect. The threshold doses of adenosine monophosphate, adenosine diphosphate and adenosine triphosphate were about 0.5 μg, 1 μg and 1 μg respectively/u. substance P. Adenine had negligible activity. The guinea-pig ileum is sometimes less sensitive to these adenosine compounds, but results are variable. In experiments on the fowl rectal caecum it is particularly important to overcome the inhibition or to separate substance P from the adenosine compounds before the assay. These substances are all likely to be present in the acetone-insoluble fraction of tissue extracts, the proportions varying with the conditions of preparation. The autolytic breakdown of adenosine triphosphate in nervous tissue produces a mixture of adenosine diphosphate, adenosine monophosphate, adenosine and adenine (Kerr, 1942). In our extracts the total purine and the adenosine triphosphate were measured, and from the low proportion of adenosine triphosphate it could be assumed that some breakdown of adenosine monophosphate to adenosine and adenine had occurred.

About 14% of the specific absorption from which the total purine was estimated is due to guanine derivatives. Therefore, guanosine, guanosine monophosphate and guanosine triphosphate were tested for pharmacological activity. Guanosine was the most active, but since it was only from 1 to 25% as active as adenosine it is not likely to be present in great enough concentrations to interfere with the assays. In any case, the responses to guanosine were abolished when the tissue was desensitized to adenosine.

The results of Thorn, Scholl, Pfleiderer & Mueldener (1958) suggest that the concentration of adenosine diphosphate would be about half that of adenosine triphosphate, but Kratzing & Narayanaswami (1953) found no adenosine diphosphate. Calculations based on the tables of Kerr (1942) suggest that significant quantities of adenosine may be present in our extracts, but the evidence is indirect. The probable concentrations of these compounds in our extracts are shown in Table 1. It can be seen that various adenosine derivatives may be present in high enough concentrations to interfere with substance P assays. It must be pointed out that these estimates refer only to our extracts. In particular, as adenosine triphosphate

TABLE 1

ESTIMATED AMOUNTS OF ADENINE AND GUANINE DERIVATIVES IN 1 MG OF ACETONE POWDERS OF BRAIN, AND IN TERMS OF SUBSTANCE P CONCENTRATIONS ASSUMING 0·2 U. SUBSTANCE P/MG ACETONE POWDER

AMP=adenosine monophosphate; ADP=adenosine diphosphate; ATP=adenosine triphosphate

Compound	Method	mole/mg	μg/u. substance P		
			Present	Test dose	Threshold
ATP	Firefly method	1·5–3	5	5	1
ADP	Kratzing & Narayanaswami (1953); Thorn *et al.* (1958)	0–1·5	5	5	1
AMP	Calculated from proportion of ATP using data by Kerr (1942)	12	17	20	0·5
Adenosine				10	0·25
Adenine				10	—
Guanine derivatives	Heald (1960)	2·5	7·5	5	—
Total	Absorption 260 mμ	17·5	—	—	—

decreases (during further autolysis) the concentration of the other adenosine compounds would be expected to rise.

At first we attempted to separate substance P from these substances by various techniques (paper chromatography, paper electrophoresis, barium and alcohol precipitation), but the recovery of substance P was not quantitative. Another method recently proposed by Laszlo (1960) involves the inactivation of adenosine monophosphate with adenylic deaminase. When adenosine monophosphate is responsible for most of the inhibitory activity of extracts this method is satisfactory, but our extracts contained sufficient adenosine triphosphate to affect the fowl rectal caecum and adenosine triphosphate is not destroyed by the enzyme.

The method adopted in our experiments was that of specific inhibition of response by high doses (specific tachyphylaxis) (Gaddum, 1953a). In general it was found that a large dose (200 μg to 2 mg) of adenosine monophosphate always desensitized the tissue to adenosine and to adenosine monophosphate, whereas adenosine triphosphate (100 μg to 200 μg) desensitized the tissue to adenosine triphosphate and adenosine diphosphate. The most certain method of abolishing the responses to all four adenosine compounds was to use a mixture of adenosine monophosphate (100 μg to 200 μg) and adenosine triphosphate (50 μg to 100 μg). However, this was not always necessary, since on many occasions adenosine monophosphate or adenosine triphosphate alone were effective against all the test substances. The large doses were put into the 4 ml. bath each time the Tyrode was renewed, and the criterion of satisfactory desensitization was that a dose of substance P should produce identical contractions when given alone and with the test substances in at least the proportions (test doses) shown in Table 1. This is illustrated in Fig 4. In this experiment addition of 1 μg adenosine triphosphate abolished the response to the test dose of substance P. The response to a mixture of the four adenosine compounds and substance P was a relaxation so large that it was not recorded. After desensitizing with adenosine monophosphate, the contractions in response to substance P were the same height whether the adenosine derivatives were added or not. This

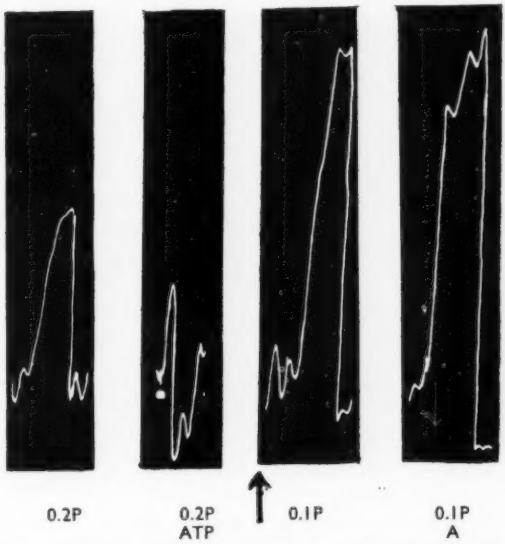

Fig. 4. As Fig. 1. Dose of substance P in u. ATP, 1 μg adenosine triphosphate was added to the bath with substance P. At the arrow adenosine monophosphate 5×10^{-5} was added and the concentration maintained throughout. A, The following mixture was added with substance P: adenosine (1 μg), adenosine monophosphate (2 μg), adenosine diphosphate (0.5 μg) and adenosine triphosphate (0.5 μg).

result is typical of about half the experiments with adenosine monophosphate. On other occasions adenosine triphosphate caused a contraction in the presence of adenosine monophosphate, and it was then necessary to use a mixture of adenosine monophosphate and adenosine triphosphate for desensitization.

Similar results were obtained with adenosine triphosphate alone. Usually the responses to adenosine monophosphate and adenosine were abolished as well as those to adenosine triphosphate and adenosine diphosphate, but in some experiments adenosine monophosphate and adenosine still caused slight inhibition in the presence of adenosine triphosphate. Addition of adenosine monophosphate to the desensitizing dose of adenosine triphosphate then abolished responses to all the adenosine compounds. Large doses of adenosine (100 μg) were as effective as adenosine monophosphate against adenosine itself and usually also against adenosine monophosphate. However, the responses to adenosine triphosphate and occasionally also to adenosine monophosphate were reversed so that a small dose produced a contraction in the presence of adenosine. Therefore adenosine could not be used alone for assay of extracts. Mixtures of adenosine (100 μg) and adenosine triphosphate (100 μg), however, were effective against all four compounds.

From these results it can be concluded that it is possible to desensitize the tissue to adenosine and its phosphates by specific tachyphylaxis. The composition of our extracts necessitated testing for sensitivity to both adenosine monophosphate and adenosine triphosphate. It would have simplified the experimental procedure

if no breakdown of adenosine triphosphate had occurred or alternatively if autolysis had proceeded to the point where no adenosine triphosphate or adenosine diphosphate remained.

The use of adenosine compounds for specific tachyphylaxis had the additional advantage of facilitating assays by inhibiting spontaneous contractions and increasing the sensitivity to substance P. When adenosine, adenosine monophosphate or adenosine triphosphate was added to the bath the caecum relaxed and remained quiescent at the greater length. It was stable for several hours. The responses to substance P from this new baseline were usually greater than before adding the nucleotide as illustrated in Fig. 4.

Assays

The approximate strength of an unknown solution was first determined by matching contractions produced by standard substance P. When the amount of material was sufficient, a 6-point assay design (Burn, Finney & Goodwin, 1950) was used. Solutions were prepared, 3 each of test and standard, so that the different doses were contained in the same vol. (usually 0.1 ml.). This was a necessary precaution, because the height of contraction varied slightly when the same dose was given in different vol. The 3 doses of each were equally spaced on a log. scale and were in the ratio 1:1.5:2.25 or 1:2:4. The 6 solutions were then given in random order according to a 6×6 Latin square. A strict time schedule was found to reduce the error of the assay. The cycle occupied from 2.5 to 4 min and substance P was allowed to act for 30 to 40 sec on different occasions. The heights of contraction from the baseline were measured and analysed according to Burn *et al.* (1950).

The dose-response relationship was investigated using 7 doses of substance P, each given 6 times in random order. The mean response was related to the log-dose by a sigmoid curve which was approximately linear over the range 0.1 u. to 1.6 u. substance P.

Two mixtures of substance P and adenosine monophosphate in the proportion 5 u. substance P to 100 μg adenosine monophosphate were made up by an assistant so that their strength was unknown to the tester. The results were calculated after statistical analysis (see Table 2). In Expt. 3 the correct value of the potency ratio

TABLE 2

RESULTS OF SUBSTANCE P ASSAYS

X indicates deviation from linearity ($P=0{\cdot}05$); + indicates deviation from parallelism ($P=0{\cdot}05$). The "result" for Expts. 3 and 4 represents the estimated relative potency (true figures 0·667 and 1·0 respectively), and for Expts. 5 to 9 represents substance P u./mg powder

Expt. no.		Doses	Groups	Deviations	Result	Fiducial limits $P=0{\cdot}05$
1	Standard	7	6	X	—	—
2	Standard	4	8	X	—	—
3	P and AMP mixture	6	6	X+	0·63	(0·56–0·70)
4	P and AMP mixture	6	6	X	1·02	(0·94–1·10)
5	Sheep brain	6	4	—	1·0	(0·89–1·14)
6	Sheep brain	6	6	—	1·39	(1·18–1·64)
7	Rabbit gut	6	6	X+	0·34	(0·29–0·40)
8	Rabbit brain	6	6	—	0·31	(0·26–0·37)
9	Rabbit brain	6	5	X	0·28	(0·25–0·31)

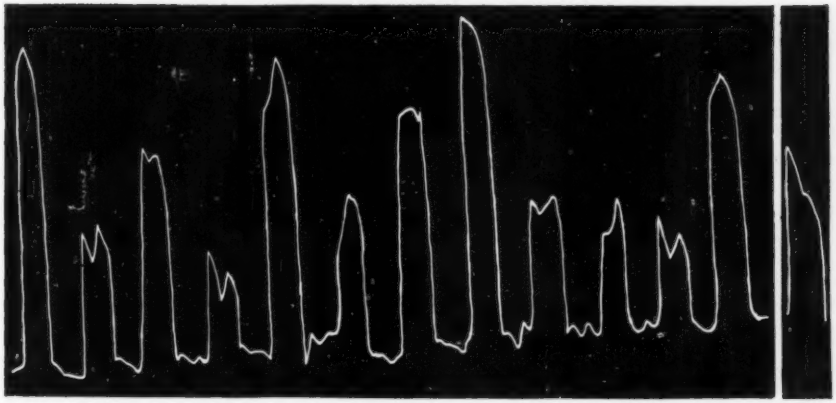

Fig. 5. As Fig. 1. E=an extract of rabbit intestine (mg). HT=5-hydroxytryptamine (ng). Doses of substance P in u.

was 0.667, so that the error was -5.5%, and in Expt. no. 4 the correct value was 1 and the error $+2\%$. These results are well within the fiducial limits. This shows that it is possible to assay substance P in the presence of adenosine monophosphate.

Six-point assays were also carried out with extracts from sheep brain, rabbit brain and rabbit intestine. One of these assays is illustrated in Fig. 5, where it can be seen that the shape of the contractions in response to extract and substance P is the same. No methysergide was used in this experiment and the caecum was unusually sensitive to 5-hydroxytryptamine, which caused a contraction in a dose of 43 ng. It can be seen that the response to 5-hydroxytryptamine was a different shape from the response to substance P and to the extract. Nevertheless, it was thought necessary to determine the 5-hydroxytryptamine content of the extract. This was done with the rat fundal strip (Vane, 1957). The extract was assayed against 5-hydroxytryptamine, substance P and a mixture of 5-hydroxytryptamine and adenosine monophosphate to allow for the depressing effect of adenosine monophosphate in the extract. The concentrations of adenosine triphosphate and adrenaline in the extract were shown to be too low to affect the assay.

It was found that the extract contained an exceptionally high concentration of 5-hydroxytryptamine (10 ng/mg). In spite of this and the unusual sensitivity of the rectal caecum to 5-hydroxytryptamine the maximum contribution of 5-hydroxytryptamine was 4.6% of the total activity on the rectal caecum. This is within the limits of experimental error. In most experiments the rectal caecum was much less sensitive to 5-hydroxytryptamine, and the effect of the 5-hydroxytryptamine even in this extract would have been negligible.

The results of these assays are given in Table 2. It can be seen that the two assays of extracts from the same rabbit brain powder agree well. In these assays the caecum was insensitive to both adenosine monophosphate and triphosphate. The agreement between the assays for the sheep brain powder is not so good. The

discrepancy between them is just large enough to suggest that the calculation of the error of the test does not take all relevant factors into consideration. If the correct result was the geometric mean of the two estimates (1.18), both of these would be near the limits of their fiducial range ($P=0.05$). This could be accounted for by pipetting errors. On the other hand, these assays, which were the first in the series, were done without checking that the tissue had been desensitized to adenosine triphosphate, since at that time it was not realized that adenosine triphosphate effects were not always abolished by adenosine monophosphate alone. It is possible that this contributed to the discrepancy in the results.

The index of precision (λ) is estimated from the ratio s/b (Gaddum, 1931, 1953b), where s is the standard deviation of a single response, calculated from the analysis of variance after eliminating components due to differences between groups of results, preparations, regression, curvature, etc. It gives an absolute estimate of the precision of the method, which is independent of the number of responses measured, and the units in which they are measured.

Woolf has pointed out that, for many purposes, it is more convenient to calculate L ($=b/s$), which is the reciprocal of λ, and this is shown in Table 3. This

TABLE 3

ESTIMATES OF ERRORS

The figures in brackets give approximate estimates made by the simple method described in the text. b=Slope (mm on drum for 10-fold dose range); R=potency ratio (estimated/assumed); s=standard deviation of a single response (mm); L=b/s=1/λ=1/index of precision; FR=fiducial ratio (upper fiducial limit/R, $P=0.05$)

Expt. no.	b	R	s	L	FR
1	49	—	5·34 (4·6)	9·2 (10·6)	—
2	130	—	4·41 (4·3)	29·4 (30·3)	—
3	80	0·63	4·68 (5·3)	17·2 (15·3)	1·12 (1·11)
4	99	1·02	4·89 (5·8)	20·2 (17·1)	1·08 (1·09)
5	101	1·00	6·90 (6·6)	14·6 (15·0)	1·14 (1·14)
6	71	1·74	5·51 (5·1)	12·8 (14·1)	1·17 (1·12)
7	79	1·48	7·69 (7·8)	10·2 (10·0)	1·18 (1·17)
8	77	1·30	8·28 (7·8)	9·3 (9·8)	1·19 (1·17)
9	123	0·94	7·35 (7·9)	16·8 (15·6)	1·11 (1·11)

suggestion was mentioned by Gaddum (1953b), but Woolf's paper on the subject was not published. In a uniform series of tests L is normally distributed, and its deviation from the true L is due only to sampling error. Since the standard deviation of $b=s_b=\frac{s}{\sqrt{S(x-\bar{x})^2}}$ and $L=\frac{b}{s}$, the standard deviation of $L=s_L=\frac{1}{\sqrt{S(x-\bar{x})^2}}$. This quantity depends only on the design of the test and is independent of b and s.

For ease of calculation, it may be assumed, as an approximation, that the tests shown in Table 2 were all 6-point assays, based on 36 results, with a dose interval of log. 2. In such tests there are 24 results for which $(x-\bar{x})$ is log. 2, and 12 for which it is zero.

$$\therefore s_L = 1/\sqrt{24(\log.\ 2)^2}$$
$$= 0.68.$$

The standard deviation of the observed values of L was much larger, namely, 6.5. This shows that the true value of L varied from test to test and that the variation

was not all due to the sampling error. Some experiments were thus actually less reliable than others, as was perhaps fairly obvious.

The mean value of L was 15.5 and the corresponding value of λ is 0.066. This result can be regarded as fairly satisfactory. In the table given by Gaddum (1953b) for experiments on isolated tissues, L varies from 5, for assays of oxytocin on the guinea-pig uterus, to 30 for assays of histamine on the guinea-pig ileum. In some of the assays by Mogey, Trevan & Young (1949) of D-tubocurarine on rat diaphragm, the value of L was as high as 63, but the value for the assay of adrenaline on rat uterus (Gaddum & Lembeck, 1949) was 16, which is similar to the mean value given by the results described here.

In the present work it was not always easy to discover the linear part of the dose-response curve, and several assays showed significant deviations from linearity. This was probably because the lower end of the curve was used in an attempt to obtain high sensitivity. The departure from strict linearity has little effect on the estimated strength of the unknown and is relatively unimportant for practical purposes. It does, however, affect the fiducial limits. Deviation from parallelism is bound to occur when there is deviation from linearity unless the doses of unknown and standard are equal. In none of the experiments was there any significant difference in curvature of the dose-response relationships for standard and unknown.

The error of these tests has been estimated on the assumption that the dose-response relation is linear and is shown in the last column of Table 3 in the form of the fiducial ratio ($P=0.05$), which is equal to the ratio of the upper fiducial limit to the result of the test (R).

The figures in brackets give estimates made by much simpler methods, which provide a useful check on the complicated calculations required for the ideal solution. Each value of s was estimated by dividing the range of the responses to each dose by the appropriate factor. With small groups this estimate is almost as reliable as the conventional method (Gaddum, 1953c). The main disadvantage of it for the present purpose is that it includes error due to differences between groups of results.

The approximate value of the fiducial ratio was calculated from the approximate value of L by the formula: Log (fiducial ratio)$=2t/L\sqrt{N}$ (where t is Student's t and N is the total number of observations). This formula, which was given in a slightly different form by Gaddum (1953b), is generally applicable to symmetrical parallel line assays. It gives a minimal estimate and is only exactly true when the mean effects of the standard and unknown are equal and when g is negligible.

The true value of the fiducial ratio will be greater than this approximation, but, in the experiments described here, the difference was negligible, except in Expt. no. 6, where R was particularly high (1.74).

The accurate method is laborious and gives scope for error. These approximate methods provide a check on the results.

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A COMPARATIVE STUDY OF STAPHYLOCOCCUS HAEMOLYSINS AND COLISAN

BY

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Staphylococcus β-haemolysin exhibits the same broad spectrum of antagonistic activity against smooth muscle stimulants as described recently for Colisan. The antispasmodic effect develops slowly and can be removed only by repeated washing. The pattern of *haemolytic* activities of Colisan is characteristically different from that of β-haemolysin, but resembles closely α-toxin. Both staphylococcus toxins can destroy paramecium and entamoeba, but the ratio of the titres at which they kill paramecium and entamoeba respectively (P:A value) is different. The P:A value of β-haemolysin is similar to that of Colisan.

In a recent publication, the spasmolytic effect of a bacterial extract ("Colisan"), prepared from a new mutant (RB-103) with antibiotic activities against certain bacteria and protozoa, was described (Bergmann, Reitler, Chaimovitz & Bergmann, 1960). It was then mentioned that the only other bacterial principle with similar pharmacological properties appeared to be staphylococcus β-haemolysin (Anderson, James & Marks, 1954; Kelsey & Hobbs, 1954). A more detailed study of this toxin was therefore undertaken, which revealed antagonistic activities against various smooth muscle stimulants, very similar to those observed with Colisan. This resemblance led us to search also for possible antiprotozoal effects of the β-haemolysin, analogous to those of Colisan.

METHODS

Staphylococcus α- and β-haemolysins. The commercial products of the Wellcome Research Laboratories, Beckenham, containing respectively 4.3 and 0.08 international units (i.u.) per mg, were used. They contained varying amounts of protein, originating from the culture medium. Throughout the terms staphylococcus haemolysins and toxins are used synonymously.

An extract of the mutant RB-103 (Colisan) was obtained through the courtesy of Hillel Remedies Inc., Haifa, Israel.

Haemolytic titre. (a) For β-haemolysin, the short "hot-cold" method of Jackson & Mayman (1958) was used with the following modified buffer: sodium chloride, 8.0 g; potassium chloride, 0.2 g; disodium hydrogen phosphate, 1.15 g; potassium dihydrogen phosphate, 0.2 g, dissolved in 800 ml. of water, were mixed with calcium chloride, 0.1 g in 100 ml., and magnesium chloride hexahydrate, 0.1 g in 100 ml., to give 1 litre of buffer of *p*H 7.0. Sheep erythrocytes, washed thrice with 0.9% sodium chloride solution, adjusted to *p*H 7.0, were suspended in this medium to give a final concentration of 1% by volume. A suitable amount of haemolysin was added and the mixture was incubated first at 37° C for

25 min and then at +4° C for 15 min. The cells were separated by centrifugation at 2,500 rev/min and the supernatant fluid examined with a Klett-Summerson photoelectric colorimeter, using filter no. 54. The same suspension without haemolysin served as blank. Percentage haemolysis was calculated by comparison with a solution, obtained by complete haemolysis of a 1% suspension of sheep erythrocytes in distilled water. Between 20 and 80% haemolysis, a linear dependence on haemolysin concentration was observed. From the appropriate plot, the concentration producing 50% haemolysis ($=H_{50}$) was evaluated as 5.3×10^{-3} i.u./ml. (b) The activity of α-haemolysin was determined on rabbit erythrocytes, according to Jackson & Little (1957). The H_{50} value, defined as above, was equivalent to 0.27 i.u./ml. (see Fig. 4a).

Amoebicidal titre (=A-titre). Strain no. 17 of *Entamoeba histolytica*, obtained through the courtesy of Professor S. Adler, Department of Parasitology, was grown in the following medium:

(a) Bacto-Peptone (Difco), 10 g; Bovril (beef extract), 3 g; sodium chloride, 5 g; disodium hydrogen phosphate, 2.5 g; water to 1,000 ml. The *p*H of this mixture is usually near 7.0. The solution was boiled to dissolve all components, filtered and sterilized at 15 lb. pressure for 30 min.

(b) Fresh beef blood was kept in the refrigerator for 24 hr and then filtered through gauze. The filtrate was centrifuged in a cold room for 15 min at 10,000 rev/min and the supernatant passed through a Seitz filter into a sterile vessel. It was then incubated at 60° C for 30 min and subsequently stored in a refrigerator.

Solutions (a) and (b) were mixed in a ratio of 10:1. To this mixture were added a few grains of sterile rice starch and a suitable size of inoculum, containing trophozoites from a 2 to 4 days' old culture (usually 0.1 to 0.2 ml. of inoculum/5 ml. of culture medium). Drugs were added simultaneously with the inoculum.

Amoebicidal tests give widely varying results if the conditions of the experiment, the composition of the medium or the strain are varied. The figures reported in this paper were obtained under strictly identical conditions; however, activity of an extract is only of value for comparison with other biological effects of extracts from the same organism.

Microscopical examinations were performed daily. When no trophozoites could be detected, a subculture was made after 48 hr. The subculture again underwent daily examination and, if no trophozoites could be found, a second subculture was initiated after an additional 48 hr. Only if the second subculture remained negative during 2 days (that is, after a total observation time of 6 days) was the absence of trophozoites assumed to be established.

Strain 17 of *Entamoeba histolytica,* which was isolated originally from the faeces of a patient, contained a single species of gram-negative rod. Therefore it appeared of importance to ascertain that the amoebicidal action affected the protozoal cells directly and did not act through an antibacterial effect. This was done in two ways: (a) After the trophozoites had been exterminated, the bacteria could easily be subcultured on blood agar. (b) Direct application of amoebicidal concentrations of Colisan to the bacteria, growing in the medium used for the amoebicidal tests, but lacking starch, revealed only slight inhibitory activity.

These results cannot be considered as unambiguous proof of a direct amoebicidal action, as slight interference with bacterial growth may be sufficient to prevent development of the amoebae and thus to cause their extermination. In a future paper we shall give new evidence for the direct amoebicidal activity of Colisan.

Lethal action against Paramecium caudatum (=P-titre). A hay infusion (1 g of hay in 200 ml. of tap water) was sterilized at 15 lb. pressure for 30 min and then inoculated with 5 vol. % of a paramecium culture, about 4 days of age. Microscopical examination at low magnification was performed after 3, 24 and 48 hr. The test substances were added in solid form or dissolved in sterile tap water. As with entamoeba, the minimum lethal dose was used for comparison of activities.

Tests on guinea-pig or rabbit ileum. The method used previously (Bergmann *et al.*, 1960) was again applied. The intestinal loops were incubated with the haemolysin for 7 min before

a smooth muscle stimulant was added, as this is the minimum interval to give the maximal antagonistic effect. The concentration of inhibitor, reducing the height of contraction by 50%, was determined for each stimulant. Between subsequent applications of a stimulant, 3 washings with Locke solution were performed in the course of 3 min.

RESULTS

Antagonism between β-haemolysin and various smooth muscle stimulants. It has been reported by Anderson, James & Marks (1954) and by Kelsey & Hobbs (1954) that staphylococcus β-toxin paralyses the spontaneous peristalsis of rabbit intestine, after an initial contraction. β-Haemolysin likewise blocked the effect of acetylcholine. Our own tests were carried out with guinea-pig ileum. Here again, the preparation of β-haemolysin at our disposal caused first a strong contraction, followed by a gradual relaxation. The initial contraction may be due to the presence of peptone or other components from the culture medium. A decisive answer can only be given when chemically pure β-haemolysin is available. On the other hand, contamination of the β-toxin by α-toxin is improbable, in view of the practically negligible action of the preparation on rabbit erythrocytes (see Table 2).

After 7 to 8 min incubation with β-haemolysin, the effect of smooth muscle stimulants was reduced to various degrees. β-Haemolysin was antagonistic to all stimulants tested (acetylcholine, 5-hydroxytryptamine, histamine, nicotine and barium chloride) (Figs. 1 and 2).

Only after the first washing did the full inhibitory activity of the toxin become apparent. This—partial or complete—block could be removed only slowly by repeated washings, recovery taking about 24 to 40 min. A similar behaviour was observed with rabbit intestine.

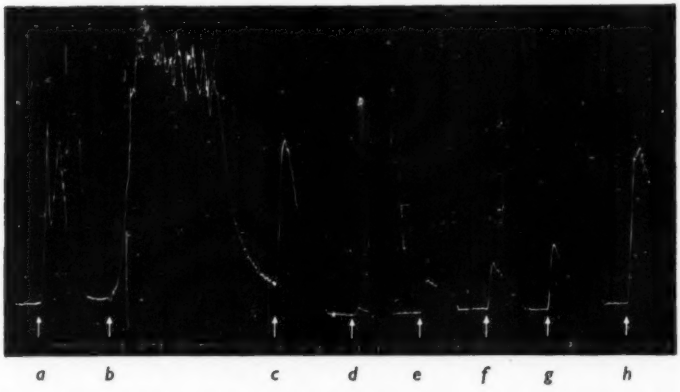

Fig. 1. Antagonism of β-haemolysin and 5-hydroxytryptamine on the isolated guinea-pig ileum. The intestine was stored in the refrigerator for 24 hr prior to use. The standard test dose of 5-hydroxytryptamine, used throughout, was 0.3 μg/ml. At a, 5-hydroxytryptamine; b, β-haemolysin, 0.08 i.u./ml.; c, 8 min later, without washing, 5-hydroxytryptamine was added. The tests with 5-hydroxytryptamine were repeated from d to h. Time intervals: c to d, 3 min; d to e, 4 min; e to f and f to g, 5 min; g to h, 10 min.

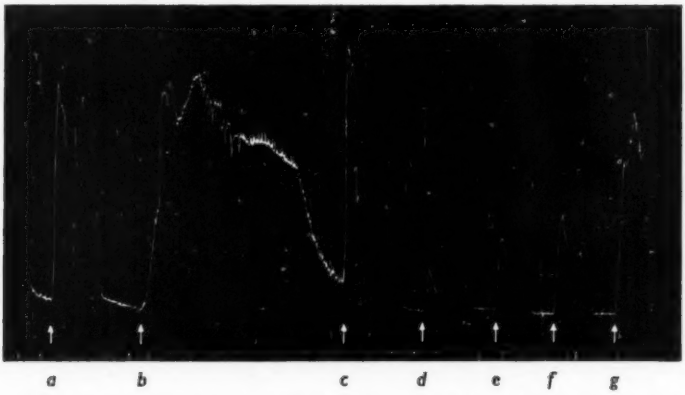

Fig. 2. Suppression of barium-chloride-induced contractions of the guinea-pig ileum by β-haemolysin. The intestine was stored, prior to use, in the refrigerator during 24 hr. The standard dose of barium chloride was 40 μg/ml. At a, barium chloride; b, β-haemolysin, 0.08 i.u./ml.; c, 9 min later, without washing, barium chloride was added; tests with barium chloride were repeated from d to g. Time intervals: c to d, 3 min; d to e and e to f, 4 min; f to g, 10 min.

Fig. 3 demonstrates the similarity of the time course of the inhibition for all stimulants tested. In Table 1 the relative inhibitory effect of β-haemolysin against the 5 stimulants has been calculated from experiments in which the same concentration of toxin was used. β-Haemolysin appears to antagonize 5-hydroxytryptamine and nicotine more effectively than acetylcholine.

TABLE 1

RELATIVE INHIBITORY ACTIVITY OF β-HAEMOLYSIN AGAINST VARIOUS SMOOTH MUSCLE STIMULANTS

All experiments were carried out with 0.1 i.u./ml. of β-toxin, on guinea-pig ileum, stored in the refrigerator for 24 hr. The stimulants were applied in equiactive doses

Stimulant	Relative inhibitory activity of β-haemolysin
Acetylcholine	1
Histamine	0·85
5-Hydroxytryptamine	1·35
Nicotine	1·4
Barium chloride	1·15

As with Colisan, application of a stimulant in amounts 10 to 20 times in excess of the standard dose provoked a contraction even at the height of the inhibition, thus demonstrating its competitive character.

For an understanding of the fact that full inhibition by β-haemolysin appears only after the first washing, it should be recalled that an immediate stimulant effect is observed when peptone alone is added to the organ bath, and that prompt recovery ensues when the bath fluid is replaced by fresh Locke solution. In contrast, α-haemolysin adheres to the intestine and can be removed only slowly (see Discussion).

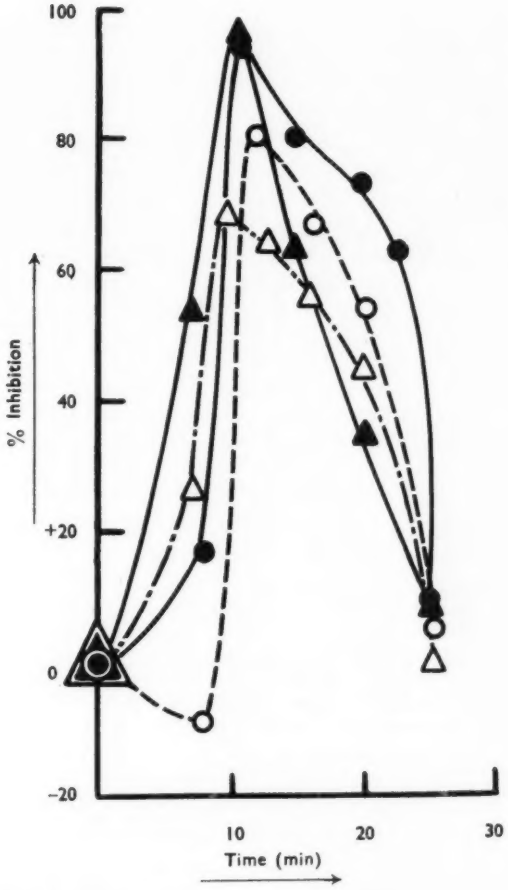

Fig. 3. Time course of inhibition of smooth muscle stimulants by β-haemolysin. In these experiments, 0.1 i.u./ml. of β-haemolysin and equiactive concentrations of the stimulants were used. The intestine was stored for 24 hr in the refrigerator before use. Ordinate: % inhibition. Abscissa: time in min. ●——●, 5-hydroxytryptamine. ▲——▲, nicotine. △——△, histamine. ○——○, barium chloride.

Comparison of the haemolytic activity of Colisan and staphylococcus haemolysins. The similarity of action of β-haemolysin and Colisan on smooth muscle suggested that these two bacterial principles may resemble each other also in other biological actions. β-Haemolysin is characterized by its inertness against rabbit erythrocytes, but it is highly active against sheep red blood corpuscles if interaction at 37° C is followed by cold incubation. However, Colisan revealed a different pattern of activity: it haemolyses sheep erythrocytes with about one quarter its efficiency

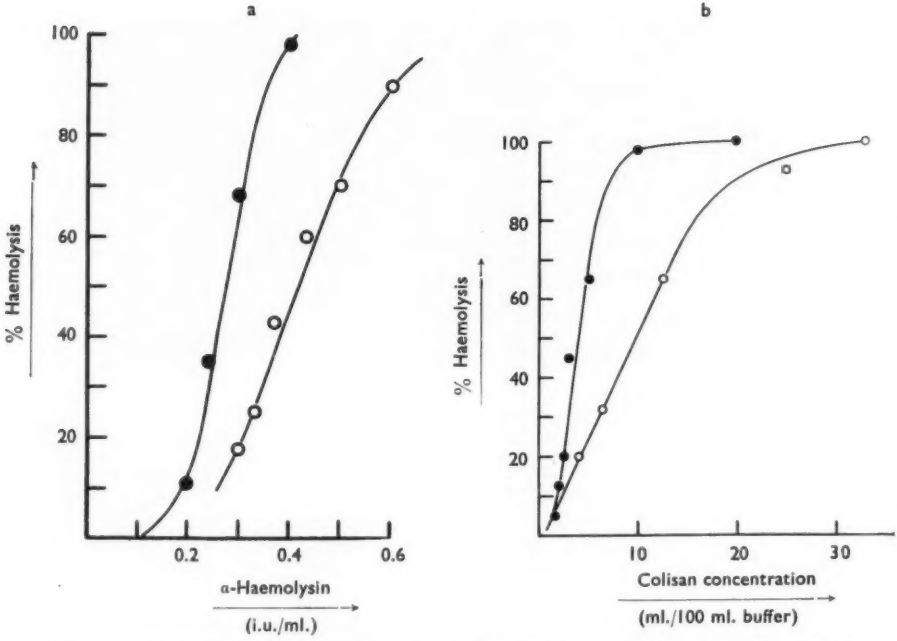

Fig. 4. Haemolytic activity against rabbit (●—— ●) and sheep (○ —— ○) erythrocytes. Incubation for 15 min at 37°. (a) Ordinate: % haemolysis; abscissa: α-haemolysin (i.u./ml.). (b) Ordinate: % haemolysis; abscissa: Colisan. From the central linear portions of these curves, the H_{50} values of Table 2 were derived.

against rabbit's cells, but the haemolytic action is not enhanced at all by subsequent cold treatment. In this respect, Colisan resembles staphylococcus α-toxin. Comparison of Colisan and α-toxin (Fig. 4) reveals indeed that in both cases the

TABLE 2

RELATIVE ACTIVITIES OF STAPHYLOCOCCUS α- AND β-HAEMOLYSIN AND OF COLISAN IN VARIOUS BIOLOGICAL TESTS

Because the bacterial extracts used do not represent pure substances, all results were expressed as % of the paramecicidal activity of the same material

Activity measured	Colisan	β-Haemolysin	α-Haemolysin
1. Lysis of *Paramecium caudatum*	100	100	100
2. Amoebicidal action against *Entamoeba histolytica*	5–10	4	25
3. H_{50}, rabbit erythrocytes	2–4	0	5,500
H_{50}, sheep erythrocytes	0·5–1·0	800	3,750
4. Antagonistic action against acetylcholine-induced contractions of guinea-pig ileum	50	50	10,000

dose–activity curve for haemolysis of rabbit cells exhibits a steeper slope than the corresponding plot for sheep erythrocytes. Likewise, the ratio of H_{50} for the two haemolytic effects is of the same magnitude (see Table 2).

Activity of haemolysins against protozoa. The above finding induced us to search for antiprotozoal activity among the staphylococcus toxins. β-Haemolysin kills both paramecium and entamoeba, the ratio of P:A titre being similar to that of Colisan. α-Toxin exhibits only weak antiprotozoal properties, but the ratio P:A ($=4$) is significantly lower than for β-toxin or Colisan (see Table 2). It is therefore improbable that the slight antiprotozoal activity of α-toxin is due to contamination with small amounts of β-haemolysin.

DISCUSSION

The experiments reported demonstrate the close similarity of action of Colisan and β-haemolysin on smooth muscle cells. The broad antagonistic spectrum of these two bacterial principles against smooth muscle stimulants supports the view that both act directly on the contractile cell. Both combine slowly with their substrate and can be removed only by prolonged washing. These features clearly distinguish β-haemolysin and Colisan from α-toxin. The latter attaches itself very quickly to smooth muscle, as contact for 30 to 60 sec is sufficient to produce the characteristic spastic contraction which passes 30 to 60 min later into a state of atony (Thal & Egner, 1961). The attachment of α-haemolysin is irreversible (Brown, Prichard & Quilliam, 1959).

On the other hand, the haemolytic properties of Colisan resemble those of α-toxin and differ from those of β-haemolysin. These differences may indicate that each bacterial extract contains a mixture of active substances which are responsible for the different biological effects. But it appears also possible that only a single active principle is present. In this case one may assume that structural differences of the active materials determine their specific mode of combination with various receptors. Only fractionation experiments will supply information on this point.

The cytocidal effect of both haemolysins and of Colisan against paramecium is much stronger than against entamoeba. This difference may—at least in part—be due to the fact that the culture medium for paramecium is essentially free from polymeric substances which could adsorb the active principles. Indeed, we have observed that the P:A value can be raised by using semisolid media for growing entamoeba instead of the liquid substrate described.

The combination of lytic effects on single cells with actions on smooth muscle organ poses the question whether a single mechanism may be responsible for these seemingly unrelated biological actions. The spasmolytic effect of Colisan and β-haemolysin develops slowly. About the same incubation period is required to immobilize paramecium by extracts with a high P-titre. It thus appears possible that in both actions a slow change of membrane permeability may take place. It should be recalled that Thal & Egner (1961) also concluded from their observations on α-toxin that it probably attacks the cell membrane directly. The present finding that protozoal cells undergo lysis, when treated with staphylococcal haemolysins,

opens a new experimental approach to the problem of the mechanism of action involved.

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Brit. J. Pharmacol. (1961), **17**, 167–175.

MECHANISM OF INCREASED SUSCEPTIBILITY TO FIBRILLATION OF THE HYPOTHERMIC MAMMALIAN HEART IN SITU

BY

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The susceptibility of auricles and ventricles to fibrillation during hypothermia of isolated hearts, and of hearts *in situ* in anaesthetized cats, was estimated by measuring the threshold for the production of fibrillation by electrical stimulation. Hypothermia reduced the threshold of *in situ* hearts, but increased the threshold of isolated perfused hearts and of heart-lung preparations. The increased sensitivity of *in situ* hearts to fibrillation could be induced by cooling the head only, and was abolished by cutting the vagi. At normal body temperature, section of the vagi had no influence on the fibrillation threshold. In contrast, cutting the sympathetic increased the fibrillation threshold of *in situ* hearts at normal temperature, but had no influence on the threshold during hypothermia. It was concluded that the increased susceptibility of anaesthetized animals to fibrillation could be attributed to changes in the central nervous control of the heart induced by the cooling of the head.

It is well known that one of the major limiting factors in the therapeutic application of artificial hypothermia is an increased susceptibility of the heart, especially of the ventricles, to fibrillation. Owing to the enhanced sensitivity to external stimuli, manipulation of the heart, which is unavoidable in heart surgery, can precipitate fibrillation (Hegnauer, D'Amato & Flynn, 1951; Bigelow, Lindsay & Greenwood, 1950). On the other hand, it has been shown by Szekeres & Lénárd (1960) that, in the isolated Langendorff preparation of cats and rabbits, susceptibility of the auricles and ventricles to fibrillation, evoked by electrical stimuli, was depressed to some extent by hypothermia. In these experiments the susceptibility to fibrillation was expressed by the fibrillation threshold, that is, by the minimal strength of current needed to produce fibrillation of the ventricles and auricles respectively. There is other evidence of a reduced susceptibility to fibrillation of the isolated heart during hypothermia. Indeed, cooling was found to be the most effective method for abolishing ventricular fibrillation produced by strong faradic stimuli or A.C. impulses in the Langendorff preparation of the rabbit heart (Dirken, Gevers, Heemstra & Huizing, 1955; Baumgartner, 1959). Prolonged fibrillation of Langendorff preparations, provoked by electrical stimulation in the presence of a reduced potassium concentration in the perfusion fluid, could not be induced in the hypothermic heart (Goodford, 1958), and the proportion of hearts fibrillating on a given calcium concentration of the perfusion fluid was reduced by cooling (Milton, 1959). Thus four independent investigations have shown a reduced susceptibility to fibrillation of the isolated Langendorff heart preparation during hypothermia.

It would be an advantage if the reason for this reduction in susceptibility to fibrillation of the isolated heart could be elucidated, for it might lead to a better understanding of the basic mechanism underlying the susceptibility to fibrillation in general, and might help towards an understanding why the mammalian heart *in situ* shows increased susceptibility during hypothermia, which would be of clinical value as well as of theoretical interest. The difference between the heart *in vivo* and *in vitro* might be occasioned by myocardial hypoxia or other metabolic deficiencies in the isolated preparation, or to extracardiac influences *in vivo*. Our present work is devoted to the investigation of differences between the behaviour of the mammalian heart in isolation and *in situ* during hypothermia. As in our earlier work, the reactions of both auricles and ventricles were considered separately in all experiments.

METHODS

Experiments were performed on 32 cats of both sexes with an average weight of 3.5 kg. The animals were anaesthetized with 1.5 ml./kg Dial-urethane solution (4% (w/v) Diallyl-barbiturate and 16% (w/v) ethyl-urethane) administered intraperitoneally. When artificial respiration just sufficient to prevent spontaneous respiration had been established, the thorax was opened by a midsternal approach. Two pairs of silver electrodes were introduced through small apertures made in the pericardium, and stitched to the ventral surface of the right ventricle and auricle respectively. The electrocardiogram was recorded by an oscillograph.

Fibrillation in both auricles and ventricles was induced by rectangular pulses of 1 msec duration and 30/sec frequency. The strength of the current passing through the heart could

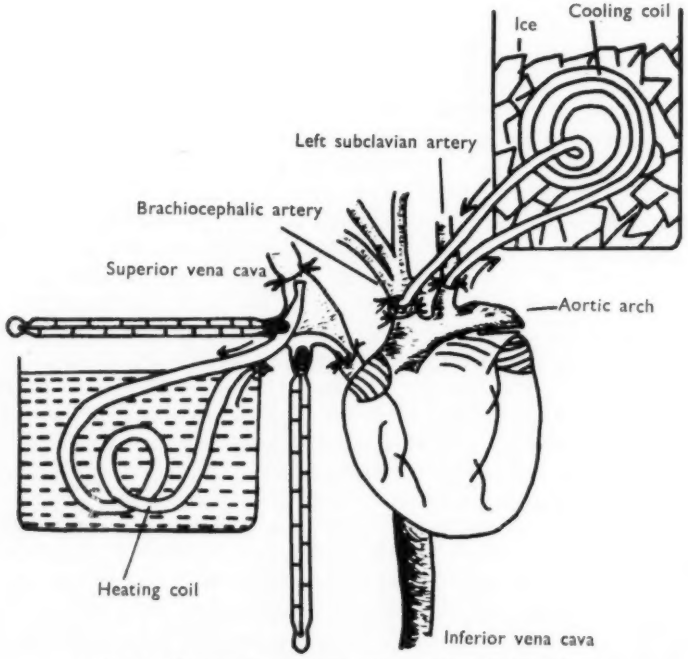

Fig. 1. Diagram of arrangement for cooling the head only.

be read directly in milliamps from a specially built oscilloscope (calibrations 1.0, 0.1, 0.05 and 0.01 mA/cm). This method was found to give reliable results in evaluating antifibrillatory drugs by DiPalma, Lambert, Reiss & Schults (1950), and had the advantage of requiring less complex equipment than techniques requiring the delivery of a stimulus during the "vulnerable period."

The temperature of the blood in the heart was measured by a thermocouple introduced into the right atrium. Hypothermia was produced either by extracorporeal cooling of the blood of the femoral artery by passing it through a polythene coil placed in ice, or in some of the experiments simply by placing ice bags into the abdominal cavity. The procedure used for isolated cooling of the head is summarized by the schematic diagram given in Fig. 1. The arterial blood flowing to the head was cooled by interrupting the brachiocephalic artery with a polythene tube immersed in ice. The venous blood returning from the head was rewarmed to body temperature similarly by a polythene rewarming coil inserted into the superior vena cava. Heart-lung preparations as described by Szegi and Rausch (1958).

RESULTS

Effect of hypothermia on the auricular and ventricular fibrillation threshold of hearts in situ *in normal anaesthetized cats.* Our first experiments were designed to investigate whether the well-known increased susceptibility to fibrillation of the hypothermic heart *in situ* could be demonstrated also by the quantitative method of determining the threshold for fibrillation by electrical stimuli. As the strength of the stimuli was increased, multiple extrasystoles, flutter and finally fibrillation appeared. The appearance of fibrillation was established (a) by direct observation of the cardiac activity, (b) by the electrocardiogram, (c) by the record of the blood pressure. Fibrillation stopped as soon as electrical stimulation was interrupted or immediately after it, as was expected in a heart as small as that of the cat. Fibrillation appeared at a well-defined and reproducible threshold. In normal anaesthetized cats the threshold for fibrillation diminished with cooling in both auricles and ventricles (Table 1). This finding, therefore, was in accord with the increased

TABLE 1

AURICULAR AND VENTRICULAR FIBRILLATION THRESHOLDS OF *IN SITU* HEARTS OF NORMAL ANAESTHETIZED CATS AT DIFFERENT BODY TEMPERATURES EXPRESSED AS A % OF THE VALUE AT 38° C

Figures represent means ± standard errors

	No. of experiments	Body temperature					
		38° C	35° C	31° C	28° C	25° C	21° C
Auricular fibrillation	7	100·0	76·6 ± 8·9	75·8 ± 0·9	70·3 ± 11·5	66·6 ± 6·6	44·5 ± 10·7
Ventricular threshold	7	100·0	73·0 ± 8·3	76·0 ± 9·4	71·6 ± 8·1	58·5 ± 7·5	60·1 ± 6·7

susceptibility to fibrillation of the hypothermic heart manipulated during cardiac surgery.

Effect of cooling on the thresholds for fibrillation of the heart-lung preparation of cats. In further experiments the Starling heart-lung preparation was used for estimation of the thresholds for fibrillation. In this preparation the heart is isolated from central influences, but is performing work under more physiological circumstances than the Langendorff preparation, and its oxygen supply is adequate for a 4 to 5 hr experiment even at 37° C. Nevertheless it reacted to cooling in the same

TABLE 2

AURICULAR AND VENTRICULAR FIBRILLATION THRESHOLDS OF THE STARLING HEART-LUNG PREPARATION OF CATS AT DIFFERENT BLOOD TEMPERATURES EXPRESSED AS % OF THE VALUE AT 38° C

Figures represent means±standard errors

	No. of experiments	Blood temperature 38° C	35° C	31° C	28° C	25° C	21° C
Auricular fibrillation	5	100·0	144·2±24·7	143·5±10·8	154·5±11·1	213·2±19·7	254·5±35·9
Ventricular threshold	5	100·0	138·1±36·2	140·4±14·7	165·5±16·1	193·0±18·2	209·0±2·13

way as did the Langendorff heart, namely, the threshold for fibrillation of both auricles and ventricles was not reduced but considerably raised by cooling (Table 2).

The influence of cooling on the threshold for fibrillation in the auricles and ventricles of the denervated heart in situ *in cats.* Hearts *in situ* in anaesthetized cats were denervated acutely, that is, both cervical vagi were cut and the stellate ganglion and the first five thoracic sympathetic ganglia on both sides were extirpated. As seen in Fig. 2, after eliminating the nervous control of the heart, the fibrillation threshold, particularly that of ventricles, increased suddenly and was no longer reduced but somewhat elevated by subsequent cooling. The susceptibility to

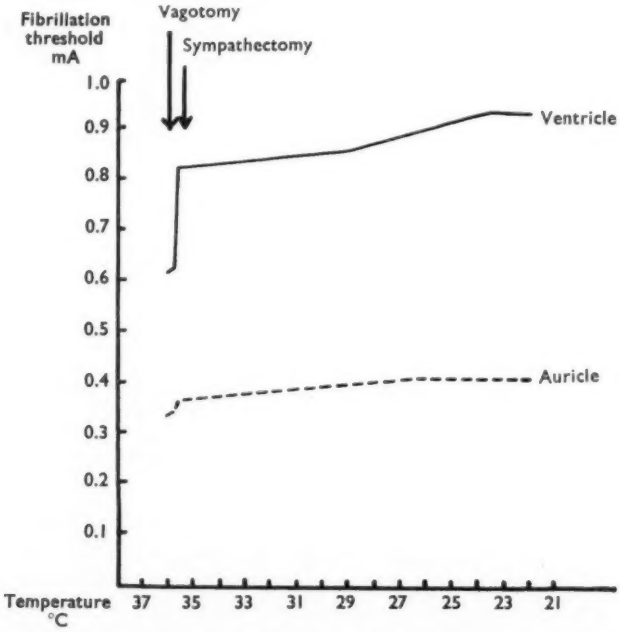

Fig. 2. The effect of cooling on the fibrillation threshold of the auricles and ventricles of the denervated *in situ* hearts of cats. Ordinate: fibrillation threshold. Abscissa: temperature.

fibrillation of the denervated heart *in situ* was thus changed by hypothermia in the same way as that of the isolated heart.

The effect of cooling of only the head on the fibrillation threshold of auricles and ventricles of the heart in situ *in anaesthetized cats.* In these experiments, the temperature of the heart and trunk was held constant throughout the experiments, but the head, whose nervous connexions with the heart were intact, was cooled gradually by the method described. As shown in Fig. 3, in spite of the unaltered

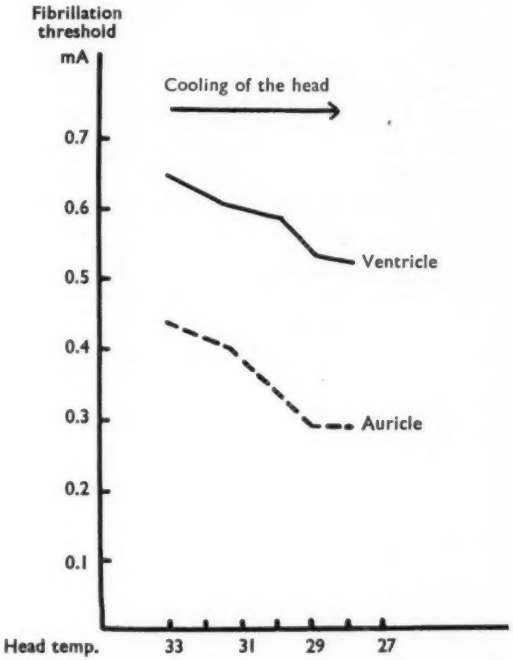

Fig. 3. The effect of cooling of the head only on fibrillation threshold of auricles and ventricles of the *in situ* heart of anaesthetized cats. Body temp., 34.5° C. Ordinate: fibrillation threshold. Abscissa: head temperature in ° C.

body and heart temperature the fibrillation threshold of both auricles and ventricles was now reduced by isolated cooling of the head, as it was when the whole animal was cooled.

Influence of the vagi on the hypothermic changes of auricular and ventricular fibrillation threshold of anaesthetized cats. When both vagi were cut in the neck (sympathetic left intact) cooling did not reduce fibrillation threshold, but both auricular and ventricular fibrillation thresholds increased (Table 3). Fibrillation appearing spontaneously in hypothermic animals could be occasionally restored to normal rhythm simply by eliminating vagal influence. Further, in the experiments

TABLE 3

THE EFFECT OF VAGOTOMY ON AURICULAR AND VENTRICULAR FIBRILLATION THRESHOLDS OF HEART *IN SITU* OF ANAESTHETIZED CATS AT DIFFERENT TEMPERATURES

Fibrillation thresholds expressed as % of the value at 38° C. Figures represent means±standard errors

	No. of experiments	Body temperature				
		38° C	35° C	31° C	28° C	25° C
Auricular fibrillation	7	100·0	108·6±4·0	106·5±8·8	114·6±7·1	149·9±8·2
Ventricular threshold	7	100·0	107·8±2·0	119·5±2·7	126·1±7·1	145·0±26·1

in which the head alone was cooled, the fall of the fibrillation threshold could be prevented by previous vagotomy, and when the vagi were cut during hypothermia the threshold for fibrillation suddenly increased above the control values (Fig. 4). When the vagi were cut at normal body temperature, the thresholds for fibrillation were not significantly changed (Table 4). The thresholds were much raised if the sympathetic nerves were cut and the vagi left intact. Sympathectomy had no effect during hypothermia (Fig. 5).

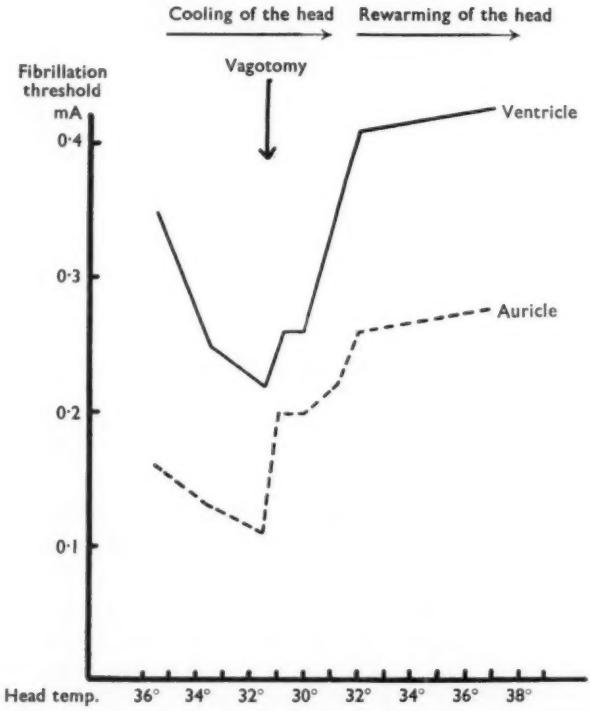

Fig. 4. Effect of vagotomy on fibrillation threshold of auricles and ventricles of the *in situ* heart if only the head is cooled. Body temp., 37° C. Ordinate: fibrillation threshold. Abscissa: head temperature.

TABLE 4

THE EFFECT OF SECTIONING OF THE VAGI AT DIFFERENT BODY TEMPERATURES ON FIBRILLATION THRESHOLD OF AURICLES AND VENTRICLES OF *IN SITU* HEARTS OF CATS

Figures represent fibrillation threshold in milliamps

			Fibrillation threshold				
		No. of experiments	Before vagotomy	After vagotomy	Difference	% difference	*P*
Auricles	37–38° C	7	0·454	0·498	0·443±0·053	+9·7	>0·50
	20–25° C	9	0·268	0·443	0·175±0·055	+65·1	>0·01 <0·02
Ventricles	37–38° C	7	0·724	0·690	0·034±0·06	−4·9	>0·50
	20–25° C	9	0·432	0·530	0·098±0·038	+22·7	>0·02 <0·05

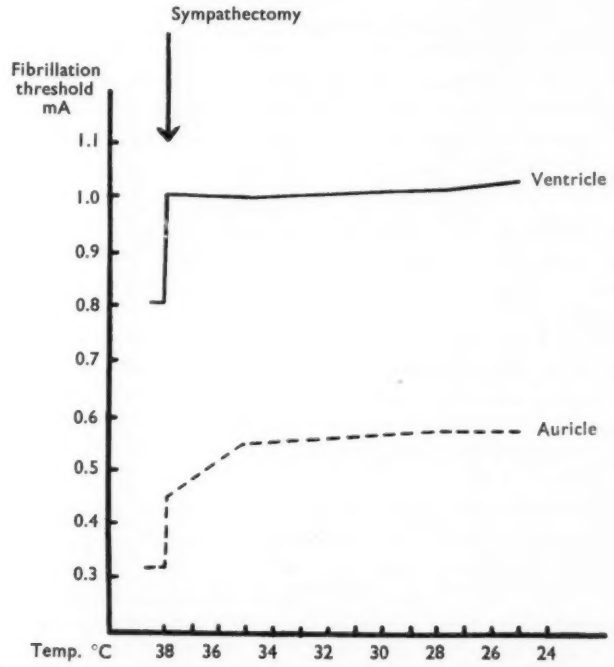

Fig. 5. Influence of sympathectomy on the auricular and ventricular fibrillation threshold of anaesthetized cats. Ordinate: fibrillation threshold. Abscissa: temperature.

DISCUSSION

There is at present no reliable method for stopping the fibrillation which may occur during hypothermia other than the rather drastic one of electrical countershock, which can be used only under suitable circumstances. It is to be hoped, therefore, that a detailed study of factors which increase or decrease the susceptibility

of the heart to fibrillation may lead to control by pharmacological means. To quote Angelakos, Laforet & Hegnauer (1957), "Experimental hypothermia, in addition to providing fundamental information needed for its successful application in surgery, can serve as an excellent experimental technique for the study of those factors which establish a condition rendering the ventricle highly susceptible to fibrillation. . . ."

The investigations reported here, carried out on the hearts of anaesthetized cats *in situ* by the fibrillation threshold method, agree with the observations made on patients, by heart surgeons, that at subnormal body temperatures manipulation of the ventricles leads much more easily to fibrillation. They also confirm the results of Covino & Beavers (1957), who found, using strong single shocks applied during the "vulnerable period" of the heart cycle, that the threshold current required to produce fibrillation of dog ventricles was lower during hypothermia. In addition our experiments have indicated that not only the ventricles but also the auricles of the heart *in situ* become more susceptible to fibrillation at lower temperatures. This observation may explain, at least in part, the arrhythmia which often precedes the onset of ventricular fibrillation.

As the threshold to fibrillation during hypothermia was raised not only in Langendorff hearts, but also in heart-lung preparations (in which there would be no reason to suppose that the myocardium was anoxic), it was unlikely that anoxia could account for the different responses to hypothermia of the isolated and *in situ* preparations.

When the head alone was cooled, the heart and trunk being maintained at normal body temperature, the fibrillation threshold was decreased, that is, the same result was obtained as when the whole animal was cooled. This suggested that impulses from the central nervous system were responsible for the difference in behaviour of *in situ* and isolated hearts. If the vagi were cut before or during hypothermia, the *in situ* heart then immediately resembled the isolated preparation, and the threshold was no longer diminished but higher than the control at body temperature. The importance of the vagi was underlined also by the observation that occasional spontaneous self-sustaining fibrillation due to cooling was often reverted to normal rhythm by section of the vagi.

The role of vagal stimulation (Winterberg, 1907) and of acetylcholine (Burn, 1960) in production of atrial flutter and fibrillation is well known, and the observations of Vaughan Williams (1958), who showed that acetylcholine increases the rate of both repolarization and depolarization of the intracellularly recorded action potential, as well as accelerating conduction velocity, provide an explanation for the action of the vagus shown here in decreasing the threshold for fibrillation.

There is, however, conflicting evidence concerning the role of cholinergic mechanisms in fibrillation. Armitage, Burn & Gunning (1957) found that in contrast to its effect on auricles, carbamylcholine failed to alter the response of the ventricles of the Langendorff preparation to stimulation and did not provoke long continued fibrillation. Moreover, Montgomery, Prevedel & Swan (1954) found that intracoronary perfusion of prostigmine to hypothermic dogs actually reduced the susceptibility of ventricles to fibrillation, but in contrast to this Scherf, Bussan, Gittinger & Torin (1955) could show no protective action by acetylcholine and

prostigmine against ventricular fibrillation elicited by focal cooling, and Malinow, Battle & Malamud (1954) found that *atropine* could prevent ventricular arrhythmias in rats.

Since cutting the vagi at normal body temperature did not influence susceptibility to fibrillation, it is probable that the difference between the behaviour of *in situ* and isolated hearts is due to an increased output of vagal impulses induced by hypothermia of the brain, though the possibility of an increased sensitivity of the heart to vagal impulses as an additional factor was not excluded. The abolition of the increased susceptibility to fibrillation during hypothermia by vagal section made it unlikely that hormonal influences were of much significance.

Section of the sympathetic increased the threshold to fibrillation in hearts at normal temperature, but had no influence during hypothermia. It is possible that the metabolic effects of adrenaline become less important on biochemical reactions already slowed down by cooling.

We wish to express our thanks to Mrs E. Udvari and Mr A. Mohos for their technical assistance, and to Dr E. M. Vaughan Williams for some assistance with the manuscript.

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ACTIONS OF TRIETHYLCHOLINE ON NEUROMUSCULAR TRANSMISSION

BY

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The effects of the triethyl analogue of choline (triethyl 2-hydroxyethyl ammonium) on muscular activity have been studied in conscious rabbits, chicks, dogs and a cat. The contractions of the tibialis anticus and soleus muscles of cats under chloralose anaesthesia, and of the tibialis anticus muscle of rabbits under urethane anaesthesia and the isolated diaphragm preparation of the rat were also used. In conscious animals, triethylcholine caused a slowly developing muscular weakness which was more severe after exercise and which resembled the symptoms of myasthenia gravis. In nerve-muscle preparations triethylcholine had a selective action in reducing the contractions of muscles elicited by a high rate of nerve stimulation while leaving unaffected the contractions caused by slower rates of stimulation. During the paralysis of the tibialis muscle of the cat produced by triethylcholine, action potentials recorded from the motor nerve were unaffected and the muscle responded normally to injected acetylcholine and to direct electrical stimulation. The failure of neuromuscular transmission produced by triethylcholine was reversed by injection of choline, but anticholinesterases were ineffective. Choline reduced the toxicity of triethylcholine in mice. It is concluded that triethylcholine produces transmission failure at the neuromuscular junction by interfering with the ability of the nerve endings to synthesize acetylcholine. The possibility that triethylcholine is itself acetylated by the nerve endings and released as an inactive neurohormone is discussed. It was shown that triethylcholine was devoid of depolarizing action and curare-like blocking action. It possesses a transient ganglion blocking action of the tetraethylammonium-type as shown in experiments in which it caused a fall in blood pressure and blocked the response of the nictitating membrane to pre- but not to post-ganglionic stimulation of the cervical sympathetic nerve.

This paper deals with observations on the pharmacological actions of triethylcholine (triethyl 2-hydroxyethyl ammonium) with particular reference to its actions at the neuromuscular junction. Triethylcholine produces muscular weakness after exercise and selectively depresses the contractions of muscles caused by high rates of nerve stimulation. These findings led to the suggestion that a substance with this type of action might be of value in the control of neurogenic spastic states (Bowman & Rand, 1961). Triethylcholine has been shown to be effective in relieving the spasm of experimental tetanus in rabbits (Laurence & Webster, 1961). Most of the previous work on triethylcholine has been concerned with its ability to replace choline in nutrition ; the substance has been shown to be capable of replacing choline in some vitaminic actions but not in others (Channon & Smith, 1936 ; Channon,

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Platt & Smith, 1937 ; Channon, Platt, Loach & Smith, 1937 ; Moyer & du Vigneaud, 1942 ; Jukes & Welch, 1942 ; Horowitz, Bonner & Houlahan, 1945 ; McArthur, Lucas & Best, 1947 ; Stekol & Weiss, 1950 ; Welch, 1950 ; Sebrell & Harris, 1954). The first pharmacological investigations were carried out by Hunt & Taveau (1909), who stated that triethylcholine lacked both the muscarinic and nicotinic actions of acetylcholine. According to Keston & Wortis (1946), acetylcholine-induced contractures of the frog rectus abdominis muscle are unaffected by the presence of triethylcholine. These authors also showed that the toxicity of triethylcholine in mice is antagonized by choline.

METHODS

Toxicity. Determinations of acute toxicity were made on male mice (A.R.C. strain) of about 20 g body weight, and in rabbits. The effects of repeated daily injections of triethylcholine were observed in 20 mice, 10 rats, 3 rabbits, 1 cat and 3 dogs.

Observations in conscious animals. In these experiments animals were exercised, restrained or observed without interference other than the injection. Rabbits and young chicks were exercised by turning them on to their backs, which procedure led to their making continual attempts to regain their normal position ; they were restrained by placing them inside a wire framework which only allowed small movements. A cat was exercised by continually moving it from a preferred position, which it attempted to regain ; it was not necessary to restrain the cat, since, when left alone, it moved infrequently. Dogs were exercised by making them follow their keeper and by playing, and they were restrained by leashing.

Observations on anaesthetized animals. Cats were anaesthetized by intravenous injection of chloralose (8 ml./kg of a 1% solution) to which pentobarbitone sodium (6 mg/kg) was added. Rabbits were anaesthetized by intravenous injection of urethane (6 ml./kg of a 25% solution).

The sciatic nerves in both legs were divided between ligatures, and bipolar platinum electrodes were placed on the peripheral portion of each nerve. The hind limbs were fixed in a horizontal position by means of steel drills through the knee and ankle joints. The tendons of both the tibialis anticus muscles and, in some experiments in cats, the tendon of a soleus muscle were detached at their insertions and tied to flat steel spring myographs. Muscle contractions were recorded on smoked paper. The nerves were stimulated by rectangular pulses usually of 10 to 100 μsec duration and of at least twice the strength required to evoke a maximal twitch. The stimulus to the nerve of one of the legs was passed through a 1:1 isolation transformer so that there was no common connexion between the electrodes to the two nerves. When required, muscles were stimulated directly with rectangular pulses of supramaximal strength and of 1 msec duration applied between the tendon and the drill in the femur. Acetylcholine was given by close-arterial injection to one tibialis muscle as described by Brown (1938). Other drugs were injected intravenously. In experiments on the knee jerk, contractions of a quadriceps femoris muscle were elicited at a rate of 1/min by tapping the patellar tendon with a Palmer automatic hammer.

In some experiments action potentials in response to nerve stimulation were recorded simultaneously from the right tibialis muscle and from the left common peroneal nerve. The cat was laid face downwards and the left sciatic nerve was fully exposed in the popliteal space and covered with liquid paraffin. Stimulating electrodes were placed as far centrally as possible, at the level of the trochanter. Nerve action potentials were recorded by means of bipolar platinum electrodes placed on the left common peroneal nerve, which was crushed peripherally to prevent the muscle action potential from complicating the recordings. Muscle action potentials in response to stimulation of the right sciatic nerve were led off from the tibialis anticus muscle by glass-mounted platinum wires inserted through the belly and tendon, or by concentric needle electrodes. Each pair of recording electrodes was connected to a differential amplifier (Tektronix type 122 battery-driven pre-amplifier) and the action potentials

were displayed on a double-beam oscilloscope (Tektronix type 502) and photographed on 35 mm film.

Respiratory movements were recorded by Gaddum's method (Gaddum, 1941). Contractions of both nictitating membranes were recorded simultaneously in cats. The pre-ganglionic cervical sympathetic nerve on one side was stimulated through bipolar platinum electrodes. The post-ganglionic fibres on the other side were stimulated by exposing the superior cervical ganglion and placing bipolar platinum electrodes so that one electrode was in contact with the ganglion and the other with the post-ganglionic fibres.

Blood pressure was recorded with a mercury manometer from a carotid artery in experiments on muscle tension and on respiratory activity, and from a femoral artery in experiments on the nictitating membrane.

Rat diaphragm. The isolated hemidiaphragm of the rat with the phrenic nerve attached was set up as described by Bülbring (1946) and suspended in McEwen's (1956) solution at 32° C in a 100 ml. bath. The nerve was stimulated by pulses of supramaximal strength and of 0.2 msec duration.

RESULTS

Acute toxicity

Mouse. The LD50's of triethylcholine chloride and of triethylcholine iodide administered intravenously, subcutaneously and orally to mice are given in Table 1. The LD50 and the 95% confidence limits were determined by the method of Litchfield & Wilcoxon (1949). Lethal doses of triethylcholine produced a slowing of respiratory movements, gasping and cyanosis, and in some of the mice there were

TABLE 1

TOXICITY OF TRIETHYLCHOLINE IN MICE

LD50 and 95% confidence limits in parentheses (mg/kg)

	Triethylcholine salt	
Route	Iodide	Chloride
Intravenous	79 (62–101)	69 (53–90)
Subcutaneous	100 (88–113)	88 (71–109)
Oral	3,900 (3,277–4,641)	—

terminal convulsions which were probably asphyxial in origin. The observations of Keston & Wortis (1946) that the toxicity of triethylcholine in mice is antagonized by choline were confirmed. Fig. 1 shows the LD50 of triethylcholine in the presence of 1/2, 1/4, 1/8 and 1/16 of the LD50 of choline chloride. The optimum protective dose of choline chloride was 100 mg/kg, which increased the LD50 of subcutaneous injections of triethylcholine chloride approximately 7-fold. We were unable to demonstrate an antagonistic action of choline on the lethal dose of triethylcholine when the compounds were given intravenously.

Rabbit. Ten rabbits were each injected with 100 mg/kg triethylcholine iodide intravenously. Five of them were continuously exercised immediately after the injection and all of these died between 6 and 17 min later. The other five were restrained ; four of these survived and one died 35 min after the injection. Death was caused by respiratory failure ; heart beats continued for 0.5 to 2 min after the last breath. The surviving rabbits developed pronounced muscular weakness which

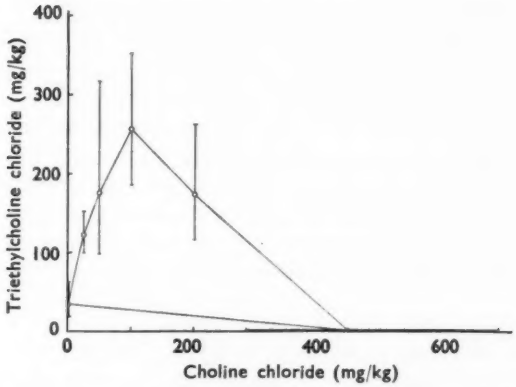

Fig. 1. The effect of choline in reducing the toxicity of triethylcholine. The circles indicate the LD50 of triethylcholine alone, of triethylcholine in combination with 4 different doses of choline, and of choline alone. The 95% confidence limits of the LD50 of triethylcholine alone and in combination with choline are indicated by the vertical lines, and of choline alone by the horizontal line. The straight line joining the LD50 points for triethylcholine alone and choline chloride alone is that to be expected if the toxicities were additive (Gaddum, 1959). All the data for this figure were obtained from one batch of mice.

was at its peak 25 to 35 min after injection ; 1.5 to 2 hr after injection full recovery had taken place, and no effect of triethylcholine was detectable even after exercise.

Chronic toxicity

The effect of daily injections of triethylcholine iodide on the growth of mice and rats is shown in Table 2. The high dose in mice, approximately 25 mg/kg, killed

TABLE 2

EFFECT OF 14 DAILY DOSES OF TRIETHYLCHOLINE IODIDE ON GROWTH IN MICE AND RATS

Mice were dosed by intravenous injection, rats by intraperitoneal injection. Asterisk denotes value significantly greater than control, $P<0.05$

Species	Daily dose/ animal	No. in group	Deaths	Initial mean wt. (g)	Mean wt. after 14 days	Mean % increase in wt.
Mouse	Control	10	0	25·8	28·0	7·6
	0·375 mg	10	1	23·9	26·3	11·0
	0·625 mg	10	5	23·0	28·6	20·2*
Rat	Control	5	1	118	213	81
	2·5 mg	5	0	116	211	82
	4·0	5	0	112	231	105*

5/10 of the group in the course of the 14 days during which the injections were given. This dose is less than half of the lower 95% confidence limit of the LD50 of single injections, which suggests that there has been a cumulative effect with injections repeated daily. The surviving mice in the high dose group and the mice and rats in the other groups were indistinguishable from the control animals in

general appearance. The mean body weight of both mice and rats in the high dose groups showed a significantly greater increase than their controls.

Three rabbits each weighing about 1.5 kg at the start of the experiment were injected with 37.5 mg of triethylcholine iodide daily for 28 days. There was no apparent deleterious effect of these injections and their final weights were each about 2.25 kg. One dog (18 kg) injected with 20 mg/kg daily for 8 days and two dogs (6 kg and 7 kg) each injected with 50 mg/kg daily for 15 and 14 days respectively showed no abnormal signs after the immediate effect of each injection had passed off. Most of the injections in dogs were made intravenously, but on some days they were given by the subcutaneous or by the intramuscular route.

The effect of triethylcholine in conscious animals

Chick. Intravenous injection of 20 mg/kg triethylcholine iodide did not cause spastic paralysis and so differed from depolarizing substances (Buttle & Zaimis, 1949). There was no immediate effect on the chick, but if it was repeatedly placed on its back it lost the ability to right itself after 15 to 20 trials ; a control chick was not fatigued by these manœuvres. After resting for 1.5 to 2 min the chick recovered the ability to stand. This sequence of fatigue and recovery could be repeated several times during the 30 min following a single injection of triethylcholine.

Rabbit. Intravenous injection of 10 to 25 mg/kg triethylcholine iodide had no apparent effect on restrained rabbits. However, if the rabbits were repeatedly exercised, muscular weakness appeared about 10 min after the injection and reached its peak after 30 min ; 90 min after injection exercise no longer produced weakness. The signs of muscular weakness were demonstrated in several ways. When a rabbit was exercised by turning it on to its back it made vigorous attempts to right itself, but after an injection of triethylcholine its movements became less vigorous until the ability to right itself was lost. If the rabbit was then allowed to rest for 1 or 2 min it recovered the ability to regain its normal posture. The weakness was confined to those muscles which were exercised. For example, if loss of righting ability was produced by continually turning a rabbit on to its left side, then a single trial by turning it on to its right side showed no impairment. In other experiments one leg was repeatedly pulled out until the rabbit lost the ability to withdraw that leg ; at this time the other legs were withdrawn normally. Withdrawal of the fatigued leg occurred after 30 to 60 sec rest. The production of muscular weakness by exercise could be repeated several times after a single injection of triethylcholine ; in the rest intervals between periods of exercise rabbits appeared normal.

Intravenous injection of larger doses of triethylcholine iodide (50 mg/kg) had more marked effects. Muscular weakness appeared after spontaneous movement on the part of the rabbit, so that the gait became incoordinated. A slight degree of head drop was present and the stance was affected. Continuous exercise caused prostration, but after resting for 2 to 5 min there was considerable recovery. Respiratory movement continued throughout. The time course of the effects was

similar to that occurring after 25 mg/kg. The effects of intramuscular injection were greater than those of the same dose intravenously.

Choline chloride (4 mg/kg) injected intravenously at the height of the response to triethylcholine caused rapid recovery to normal muscular activity. Neostigmine methylsulphate (0.15 mg/kg) did not reverse the muscular weakness.

Cat. Intravenous injections of 50 mg/kg of triethylcholine iodide (3 observations) produced an immediate relaxation of the nictitating membrane and a dilatation of the pupil. The nictitating membrane returned to normal in 10 min, but the pupillary dilatation persisted for about 1 hr. Signs of muscular weakness and disturbance in walking movements appeared 10 to 15 min after injection. Complete exhaustion after walking for a few feet was observed 25 to 35 min after injection. However, after a rest of 1 to 2 min another period of walking was possible. Small movements, such as withdrawal of the leg from a forced extension, were possible even when prostrated by exercise. Respiratory movements were always adequate. The ability to make more sustained movements returned in 35 to 40 min and full recovery in 80 to 100 min after injection.

Intraperitoneal injections (2 observations) produced the same general pattern of events, except that the weakness was more pronounced and persisted longer, so that full recovery was delayed to 150 min after injection. The pupillary dilatation and relaxation of the nictitating membrane were less marked and only occurred at the height of the signs of muscular weakness.

Dog. Intravenous injection of 20 mg/kg triethylcholine iodide had no immediate effect in dogs, but 50 mg/kg produced a relaxation of the nictitating membrane with return to normal by 10 min. When dogs were continuously exercised signs of muscular weakness appeared 6 to 10 min after the injection of 20 to 50 mg/kg, and reached a maximum after 20 to 30 min. This effect of the injection was not detectable after about 1 hr. The first sign of muscular weakness was lack of co-ordination in walking ; the head was not held erect, the lower jaw hung open and the eyelids drooped. After strenuous exercise, such as running, dogs were unable to support their own weight, although they were not paralysed since small movements were still made and respiratory movements continued. If the dogs were then allowed 2 to 3 min of rest they were again capable of a short period of strenuous exercise. Even when only lightly exercised, so that muscular weakness was not produced, triethylcholine had an effect on the behaviour of the dogs ; they frequently adopted relaxed resting positions and seemed to be disinclined to obey their keeper's orders. In this condition they alerted to stimuli such as noise and movement, but they did not stand or walk except after repeated commands from their keeper.

An intramuscular injection of triethylcholine caused a more pronounced and longer-lasting effect than an intravenous injection of the same dose.

Observations on nerve-muscle preparations

Cat. In the cat under chloralose anaesthesia, the intravenous injection of triethylcholine caused a slowly developing decrease in the size of the contractions of the tibialis and soleus muscles when the frequency of nerve stimulation was high, but

at low rates of stimulation the contractions were not affected. The higher the frequency of nerve stimulation, the greater the effect of triethylcholine in depressing muscle contractions, and the more rapid the onset of its effect. In order to study these effects, it was necessary to use a frequency of stimulation which itself did not cause fatigue of the preparation. In control experiments twitches of a tibialis muscle in response to nerve stimulation at frequencies greater than 2/sec gradually decreased in size, often to below 20% of their original level, after 2 hr of continuous stimulation. When the frequency of stimulation was 1/sec the tension of the twitches increased during the first 2 to 3 min and then remained constant. Therefore, in the majority of the experiments on the anaesthetized cat, the highest frequency of stimulation used was 1/sec.

Figs. 2, 3 and 4 show that intravenous injections of triethylcholine decreased the tension of indirectly excited maximal twitches of the tibialis anterior muscle stimu-

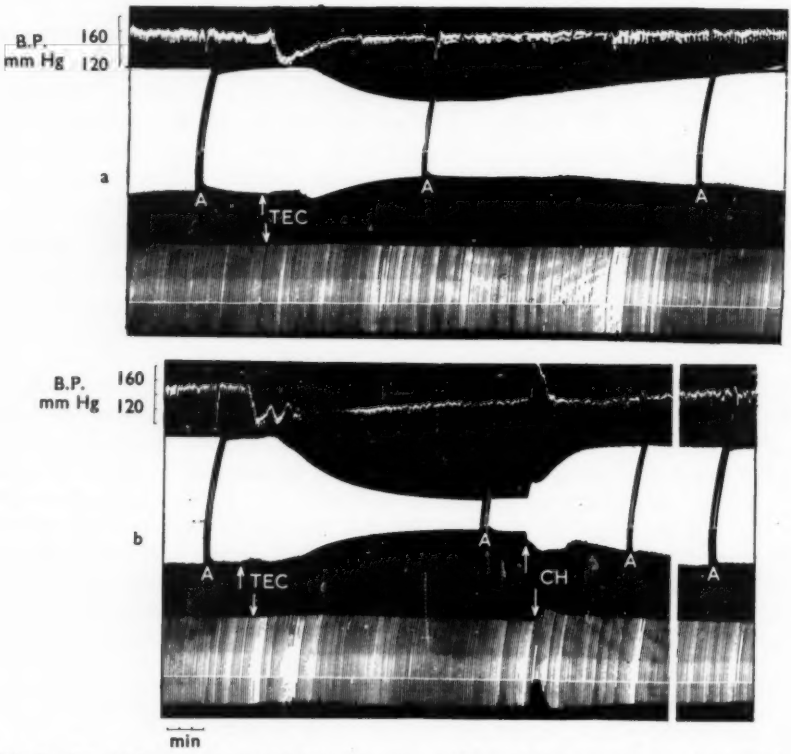

Fig. 2. Cat, 4.0 kg. Upper record, blood pressure, middle and lower records, maximal twitches of right and left tibialis anticus muscles elicited indirectly 1/sec and 1/10 sec respectively. At A, 10 μg acetylcholine was injected close-arterially to the right tibialis anticus muscle, electrical stimulation being temporarily stopped during the injection. At TEC in a, 10 mg/kg, and at TEC in b, 30 mg/kg of triethylcholine chloride were injected intravenously. At CH, 5 mg/kg choline chloride was injected intravenously.

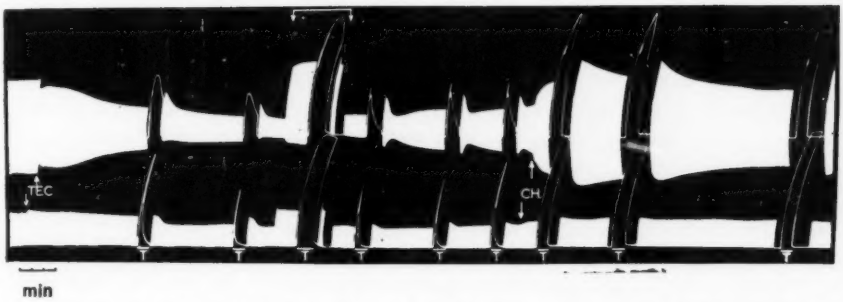

Fig. 3. Cat, 5.5 kg. Maximal twitches of the tibialis anticus (upper record) and soleus muscles (lower record) elicited indirectly once every second. At TEC, 35 mg/kg of triethylcholine iodide, and at CH, 5 mg/kg of choline chloride were injected intravenously. At T, tetani were elicited at a frequency of 50/sec for approximately 6 sec. During the tetani the kymograph speed was increased. During the period marked ⌊⌋, the muscles were stimulated directly. The animal had previously recovered from an initial injection of 35 mg/kg of TEC.

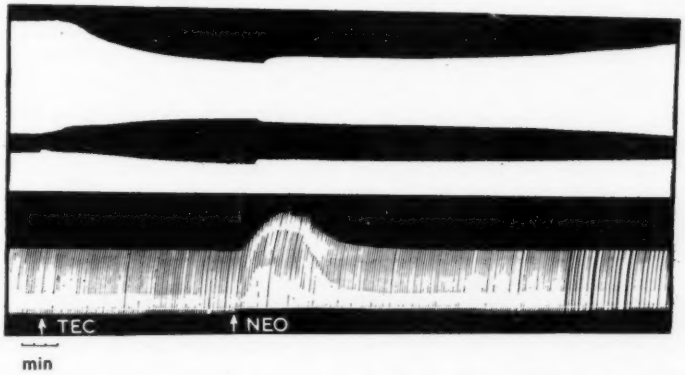

Fig. 4. Cat, 3.2 kg. Upper and middle records: maximal twitches of the right tibialis anticus and soleus muscles respectively, elicited indirectly once every sec. Lower record: maximal twitches of the left tibialis anticus muscle elicited indirectly once every 10 sec. At TEC, 30 mg/kg of triethylcholine iodide, and at NEO, 0.1 mg/kg neostigmine methylsulphate injected intravenously.

lated at a frequency of 1/sec, but the twitches of the contra-lateral tibialis, which was stimulated once every 10 sec (Figs. 2 and 4), were not affected. The time of onset of the effect of triethylcholine on muscle contractions, while dependent on the frequency of stimulation, was but little affected by increasing the doses of triethylcholine iodide from 20 mg/kg up to 50 mg/kg, and occurred 6 to 12 minutes after injection when the frequency of stimulation was 1/sec. The time from injection to the maximum reduction of contractions, and the duration of the effect, depended on the dose. After an initial injection of 40 mg/kg triethylcholine iodide the maximum degree of block occurred about 20 min after injection and the twitches

regained their normal tension 60 to 80 min later. With the second and subsequent injections of triethylcholine, some cumulative effect was seen. For example, Fig. 5 shows that a second injection of triethylcholine produced a greater degree of block with a more rapid onset.

In some experiments the first effect of an injection of triethylcholine was to produce a small increase in twitch tension, which can be seen in Fig. 2 in the record from tibialis muscle and in Figs. 3 and 4 in the records from soleus muscle. With the second and subsequent injections the initial increase was sometimes replaced by an abrupt transient depression of the twitches, which was then followed by the usual more slowly developing and longer-lasting paralysis. This is evident in Fig. 3, which illustrates the response to a second injection of triethylcholine.

With equal frequencies of nerve stimulation the maximal twitches of the tibialis muscle were always blocked to a greater extent than those of the soleus muscle. This difference is illustrated in Figs. 3 and 4. At the height of the effect of triethylcholine in another experiment the twitches of the tibialis muscle were depressed by 66.7% while those of the soleus were depressed by 27.5%.

During a partial paralysis of the maximal twitches, the tension of an indirectly elicited tetanus was markedly reduced and waned during the period of stimulation (Fig. 3). This inability of the muscle to sustain tetanic tension was more pronounced in the soleus than in the tibialis muscle, possibly because the frequencies of tetanic stimulation used (50 to 100/sec) were well above the physiological range in the case of the soleus muscle. After the tetanus the maximal twitches of both muscles were at first slightly increased in tension, but subsequently declined to a level below that before the tetanus. Fig. 5 is the record of an experiment in which contractions

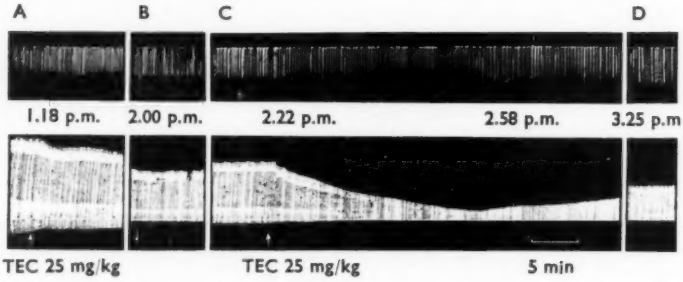

Fig. 5. Effect of triethylcholine on respiratory movements (upper) and contractions of tibialis anticus muscle (lower) in cat. Inspirations are downwards and tibialis contractions upwards. The sciatic nerve was stimulated at 100/sec for 0.2 sec every 5 sec. The doses of triethylcholine (TEC) refer to the iodide.

of a tibialis muscle were recorded in response to nerve stimulation at 100/sec for 0.2 sec every 5 sec. In the original record it can be seen that initially the tetanic contractions were well maintained, but after triethylcholine the contractions resembled twitches.

We have never observed a complete neuromuscular paralysis with doses of triethylcholine iodide up to 50 mg/kg, and the greatest decrease recorded in the twitch tension of the tibialis muscle was of the order of 80 to 90%.

At the height of a partial paralysis produced by triethylcholine, direct stimulation of the muscle produced normal contractions. Fig. 3 shows maximal twitches and a tetanus produced by direct stimulation at the time of maximum block of the indirectly excited contractions of both the tibialis and the soleus muscles. Throughout the blocking action contractions of the tibialis muscle produced by close-arterial injections of acetylcholine remained equal in tension to those produced before triethylcholine (Fig. 2).

Simultaneous recordings of muscle and nerve action potentials before and after triethylcholine are shown in Fig. 6. Triethylcholine markedly reduced the amplitude

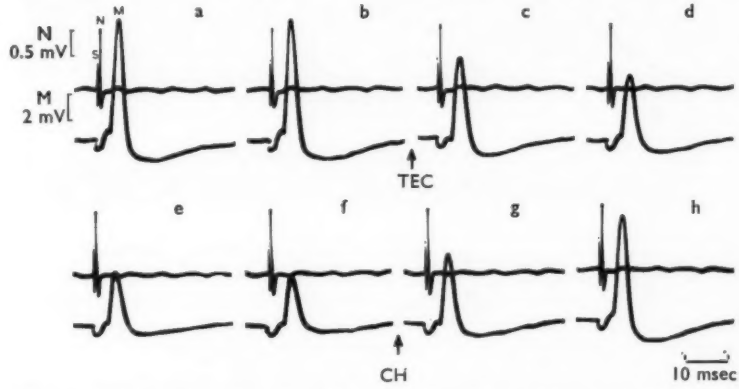

Fig. 6. Cat, 2.1 kg. Maximum action potentials recorded from the peripheral end of the left common peroneal nerve and from the right tibialis anticus muscle (concentric needle electrodes) in response to stimulation of the nerve once every second. S, Stimulus artifact; N, nerve action potential; M, muscle action potential; a and b were recorded before injection with an interval of 10 min between them; c, d, e and f were recorded 12, 14, 16 and 20 min respectively after the intravenous injection of 75 mg/kg of triethylcholine iodide; g and h were recorded 1 and 3 min after the intravenous injection of 5 mg/kg choline chloride.

of the muscle action potential, but was without effect on the shape and size of the nerve action potential. Triethylcholine iodide injected close-arterially in doses up to 5 mg did not cause contraction of the tibialis muscle.

Rabbit. In the rabbit under urethane anaesthesia, triethylcholine produced effects on the contractions of the tibialis muscle similar to those described in the cat, except that higher frequencies of stimulation were required to demonstrate them. Thus in the rabbit, short bursts of high frequency stimulation were found more suitable than single pulses. In the majority of experiments the muscle of one leg was excited every 5 sec by stimulating the nerve at a frequency of 100 or 250/sec for a period of 0.2 sec. With this type of stimulation, the contractions decreased in tension over the first 2 to 3 min and then remained constant at approximately

80% of the initial tension for several hr. The muscle of the other leg was simultaneously excited by single supramaximal shocks applied to the nerve once every 5 sec.

In the experiment illustrated in Fig. 7, triethylcholine iodide (40 mg/kg) caused an initial transient decrease in the tetanic contractions, followed by a more slowly developing block of the contractions which was more rapid in onset than that seen in the cat. The duration of the effect was similar in the two species. The slowly stimulated muscle was completely unaffected by triethylcholine.

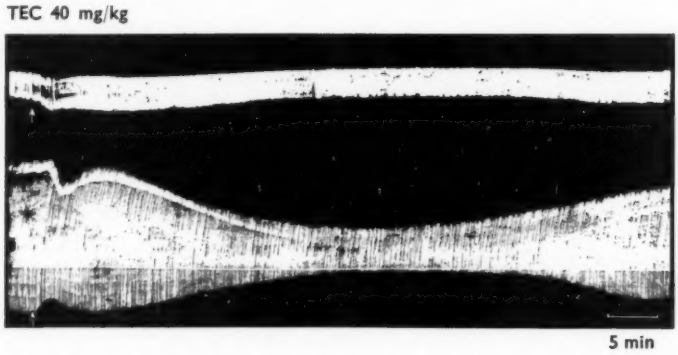

Fig. 7. Effect of triethylcholine on respiratory movements (upper trace) and contractions of tibialis anticus muscle (lower trace) of rabbit. Inspirations are downwards and muscle contractions upwards. The sciatic nerve was stimulated at 100/sec for 0.2 sec every 5 sec. The tetanic contractions of the tibialis muscle were depressed, but respiration was not impaired after the intravenous injection of 40 mg/kg triethylcholine iodide.

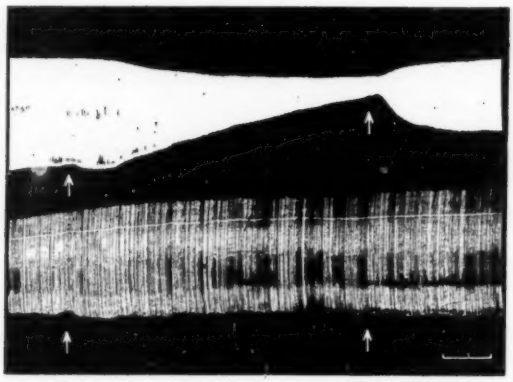

Fig. 8. Maximal twitches of 2 hemidiaphragms from one rat mounted in the same 100 ml. organ bath. Contractions are downwards. In the upper tracing the phrenic nerve was stimulated at 1/sec and in the lower at 1/10 sec. At TEC 40 mg of triethylcholine iodide and 1 hr later at Chol. 10 mg of choline chloride were added to the bath.

Rat diaphragm. The contractions of the isolated rat diaphragm to stimulation of the phrenic nerve were gradually reduced after the addition of triethylcholine to the bath when the rate of nerve stimulation was high, but were not affected by low rates of stimulation. The experiments of Fig. 8 show the contractions of both hemidiaphragms from one rat mounted in the same bath. The upper tracing, which is of a diaphragm driven at 1/sec, shows a marked reduction in contractions 1 hr after 0.4 mg/ml. triethylcholine iodide ; the lower tracing shows that triethylcholine did not reduce the contractions of a diaphragm driven at 1/10 sec. The maximum rate of nerve stimulation which did not result in a reduction of the contractions of the diaphragm in the presence of 0.4 mg/ml. triethylcholine iodide was about 1/3 sec. With frequencies of stimulation higher than 1/sec the rate of onset of failure was more rapid. The initial effect of triethylcholine on the diaphragm was to produce a slight increase in the contractions, which was followed by the slowly developing block when stimulation was rapid.

The effects of gallamine and of tubocurarine were tested on two hemidiaphragms stimulated at 1/sec and 1/10 sec in the same bath. With these neuromuscular blocking drugs the greatest effect was on muscle with a high rate of nerve stimulation, but some degree of block was detectable at slow rates. Triethylcholine, on the other hand, even in concentrations up to 5 mg/ml., which was the maximum administered, did not reduce the contractions of the diaphragm produced by slow rates of stimulation.

Effect of neostigmine and edrophonium. Neostigmine and edrophonium, in doses of 100 μg/kg and 200 μg/kg respectively, caused only a small increase in the tension of the triethylcholine blocked twitches in the cat. These doses were twice as great as those necessary completely to antagonize a similar degree of paralysis produced by tubocurarine. Fig. 4 illustrates the slight effect of neostigmine at the height of a partial paralysis produced by triethylcholine in the tibialis and soleus muscles of the cat. The small increase in twitch tension produced by neostigmine may well have been a consequence of repetitive firing of some of the unblocked fibres, rather than to an increase in the number of fibres contributing to the gross tension. Evidence in support of this was provided by the experiments on the rabbit in which tetanic contractions were recorded in place of twitches. In these circumstances repetitive firing cannot be produced and neostigmine and edrophonium were completely without effect on the partially blocked contractions.

Effect of choline. Choline antagonized the effect of triethylcholine in reducing the contractions of indirectly stimulated muscles. In both cat and rabbit the intravenous injection of choline chloride in doses of 1 to 5 mg/kg caused a striking and rapid reversal of the effect of triethylcholine (Figs. 2, 3 and 6). A similar reversal was obtained in the isolated diaphragm preparation when choline was added to the bath in a concentration of 50 to 200 μg/ml. (Figs. 8 and 9). The same amounts of choline given first prevented or reduced the development of the block produced by triethylcholine ; this effect in the rat diaphragm is illustrated in Fig. 9. With the larger doses of choline chloride (5 mg/kg) in the cat, the antagonistic action took place in two stages ; an initial abrupt but short-lasting effect was followed by a more slowly developing sustained and complete antagonism (Fig. 2). The initial

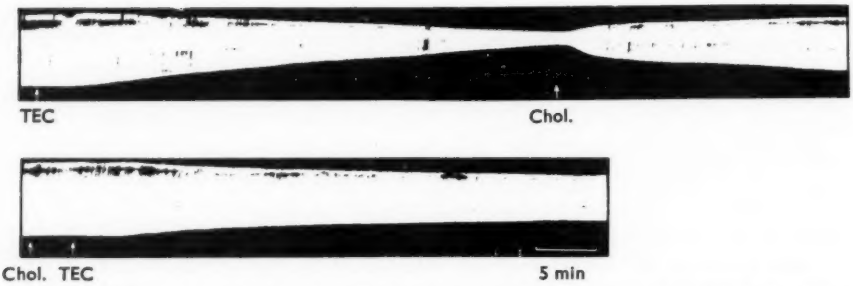

Fig. 9. Maximal twitches of isolated rat diaphragm (contractions downwards) elicited by stimulation of the phrenic nerve at a frequency of 1/sec. At TEC 200 μg/ml. of triethylcholine chloride and at Chol. 50 μg/ml. of choline chloride were added to the bath. In the upper tracing choline reversed the action of triethylcholine. In the lower tracing, which was obtained from the same preparation, choline added first reduced the action of triethylcholine.

rapid antagonism was probably caused by the direct end-plate depolarizing action of choline, since other depolarizing drugs (decamethonium and suxamethonium) produced a similar effect. With the latter drugs, however, the more sustained secondary phase of antagonism was absent. Doses of choline greater than 5 mg/kg caused an initial sharp increase in the partially blocked twitches which was often followed by a pronounced but short-lasting increase in the depth of block. The twitch tension then rapidly recovered and complete antagonism of the triethylcholine block occurred. Triethylcholine did not therefore appear to prevent the block by depolarization which large doses of choline are known to produce (Hutter, 1952). The slowly stimulated muscle on these occasions was also temporarily paralysed by choline.

During the antagonistic action of choline in the cat, contractions of the muscle produced by injected acetylcholine were regularly increased. Fig. 2 illustrates this effect which is probably a consequence of the weak anticholinesterase action of large amounts of choline (Augustinsson, 1948).

In control experiments on the cat and on the rat diaphragm, in which the nerve was stimulated at a sufficiently high frequency to cause fatigue of the contractions, choline was without any beneficial effect and, in fact, often hastened the decline of the twitch tension.

Knee-jerk. In a few experiments, knee-jerks elicited once every minute were recorded from one leg, simultaneously with maximal twitches of the tibialis muscle of the opposite leg elicited indirectly once every sec. Triethylcholine, in doses of 40 mg/kg injected intravenously, depressed the maximal twitches but was without effect on the reflexly induced contractions of the quadriceps muscle.

Comparison with hemicholinium

In the cat, hemicholinium (Schueler, 1955) was effective in smaller doses than triethylcholine, but its effects differed from those of triethylcholine in certain respects. In doses of 1 to 2 mg/kg hemicholinium dibromide caused an immediate decrease in

the contractions produced both by nerve stimulation and by the close-arterial injection of acetylcholine. This was followed by a more slowly developing decrease in twitch tension during which the response to acetylcholine returned to, and was maintained at, the control level. Choline, in doses which completely antagonized the paralysis produced by triethylcholine, caused an abrupt but only short-lasting antagonism to hemicholinium. As with triethylcholine, the soleus muscle was more resistant to the blocking effect of hemicholinium than the tibialis muscle and, like the paralysis produced by triethylcholine, that produced by hemicholinium wore off more rapidly when the frequency of stimulation was reduced. Fig. 10 illustrates these effects

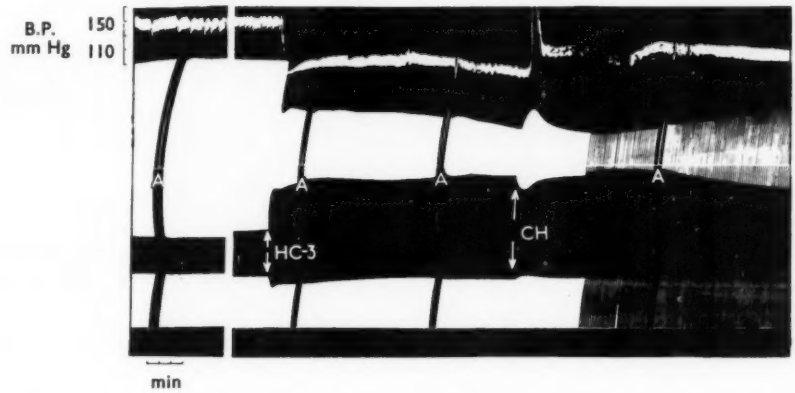

Fig. 10. Cat, 3.4 kg. Upper record: blood pressure; middle and lower records: maximal twitches of the tibialis anticus and soleus muscles respectively elicited indirectly once every second and, towards the end of the record, once every 10 sec. At A, 8 μg acetylcholine injected close-arterially to the tibialis anticus muscle. Electrical stimulation was temporarily stopped while acetylcholine injections were made. At HC–3, 2 mg/kg of hemicholinium dibromide, and at CH, 5 mg/kg choline chloride were injected intravenously.

of hemicholinium. The initial abrupt decrease in twitch tension, during which the response of the muscle to acetylcholine was depressed, was probably a consequence of the weak curare-like action of hemicholinium (Reitzel & Long, 1959a ; Schueler, 1960).

Comparisons of the relative potency of hemicholinium and triethylcholine, taking the doses of hemicholinium used by other workers (Reitzel & Long, 1959a and b ; Wilson & Long, 1959), is difficult since the effect obtained depends on the parameters of the stimulation. For example, Reitzel & Long (1959a) used stimuli of 3 msec duration, but in the majority of our experiments the pulse width was 50 μsec. On a few occasions, the pulse width was increased to 3 msec during the experiment and this caused a marked increase in the response of the muscle to a single shock applied to the nerve. Recordings of the gross muscle action potential with belly-tendon electrodes showed that the response had become markedly repetitive and under these conditions the block produced by both triethylcholine and

hemicholinium was increased. When compared under the same conditions, hemicholinium was shown to be about 20 times more potent than triethylcholine on a weight basis. However, the doses of choline required to reverse the effects were larger in the case of hemicholinium.

A further comparison between triethylcholine and hemicholinium was made on the frog rectus abdominis muscle. Hemicholinium dibromide in a concentration of 150 μg/ml. caused a 50% reduction in the response to acetylcholine, but triethylcholine iodide even in concentrations up to 5 mg/ml. did not reduce the acetylcholine responses. Triethylcholine did not itself produce contraction of the rectus abdominis muscle.

Respiratory movements

In the experiments to determine the toxicity of triethylcholine in mice and rabbits death was apparently due to respiratory failure, and in the isolated diaphragm preparation of the rat, contractions elicited by phrenic nerve stimulation were depressed by triethylcholine. Therefore experiments were carried out in which respiratory movements were recorded.

The effect of the first injection of triethylcholine in the rabbit was a slight hyperpnoea, which was probably reflexly induced by the fall in blood pressure (Fig. 7). After large doses of triethylcholine iodide (100 mg/kg) the rate and depth of respiratory movements were decreased and became erratic. These changes did not occur immediately after the injection, but developed slowly. After the injection of choline the rate and depth of respiratory movements gradually increased and became more regular.

Our previous experiments showed that the rate of failure of responses to nerve stimulation depended on the frequency of stimulation. Therefore it was important to establish the relationship between failure of a muscle contracting in response to stimulation of its nerve and the failure of contraction of the respiratory muscles in response to the respiratory drive. The experiment of Fig. 7 shows that a single injection of triethylcholine iodide (40 mg/kg) into a rabbit was without effect on respiratory movements, but markedly decreased the response of the tibialis muscle to stimulation of the sciatic nerve. A similar experiment on a cat is shown in Fig. 5. The injection of triethylcholine iodide (25 mg/kg) produced, after 40 min, a 40% decrease in the tension developed by the tibialis muscle in response to brief tetanic shocks to the nerve. At this time the only effect on respiratory movements was a slight slowing. A second injection of 25 mg/kg triethylcholine led to an 85% decrease of response of the tibialis muscle and the respiratory movements were further slowed and became erratic and smaller in amplitude. However, they were clearly sufficient to maintain life. In other experiments in which 40 mg/kg triethylcholine iodide was given there was a pronounced reduction in the contractions of the tibialis muscle, but spontaneous respiration persisted.

The depressor action of triethylcholine

The intravenous injection of triethylcholine produced a transient fall in blood pressure in rabbit, cat (Figs. 2 and 11) and dog. In the experiments on the conscious cat and dog we observed a relaxation of the nictitating membrane immediately after

the injection of 50 mg/kg of triethylcholine. Therefore it seemed likely that the depressor action of triethylcholine may have been due to ganglionic blockade, especially since triethylcholine is closely related to the ganglion blocking drug tetraethylammonium. The experiment of Fig. 11 shows that the reduction

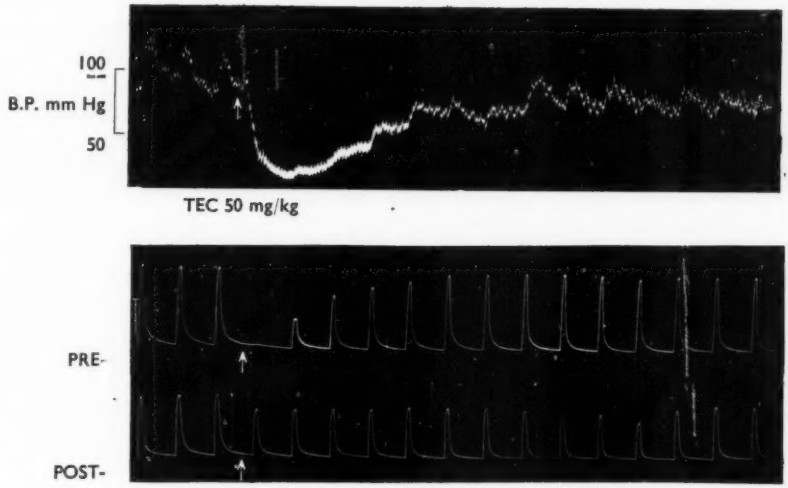

Fig. 11. Cat, 2.8 kg. Effect of triethylcholine iodide on blood pressure (upper tracing), and on the contractions of nictitating membranes in response to sympathetic nerve stimulation with 0.5 msec pulses at 20/sec for 12 sec every 2 min. The contractions of the left nictitating membrane (middle trace) are in response to preganglionic nerve stimulation and the contraction of the right nictitating membrane (lower trace) are to postganglionic nerve stimulation.

in response of the nictitating membrane to pre-ganglionic nerve stimulation was completely blocked at a time corresponding to the maximum fall in blood pressure and the recovery of response of the nictitating membrane was approximately parallel with the recovery of the blood pressure.

In the dog the pressor effect of 0.15 mg/kg of tetramethylammonium and the fall in blood pressure caused by stimulation of the peripheral end of the cervical vagus nerve were transiently blocked by 30 mg/kg of triethylcholine iodide. Intravenous injections of 10 to 30 mg/kg triethylcholine iodide produced a fall in blood pressure of 10 to 30 mm Hg. This depressor response was not blocked by atropine in doses up to 1 mg/kg, but was absent after complete ganglion block by hexamethonium bromide (4 mg/kg).

DISCUSSION

The most striking property of triethylcholine is its ability gradually to reduce the size of contraction of a muscle rapidly stimulated through its motor nerve. Thus, in experiments on nerve-muscle preparations *in situ* and in the organ bath, triethylcholine reduced the contractions in response to motor nerve stimuli at a high rate

while producing no effect where the rate of stimulation was low. Similarly in conscious animals injected with triethylcholine, muscular weakness was more pronounced after exercise, unexercised muscles being relatively unaffected. A more selective blocking action on muscles stimulated at a high rate is also seen with curare-like drugs (Preston & Van Maanen, 1953), but with these drugs the difference in effect on rapid and slow stimulation is much less marked than with triethylcholine.

Our experiments allow us to locate the site of action of triethylcholine. When muscle contractions are markedly reduced by triethylcholine, conduction in the motor nerve trunk is unaffected since the nerve action potentials were unchanged in shape and size. The muscle responded normally to direct electrical stimulation and to injected acetylcholine, which shows that the block is not due to an effect on the contractile mechanism itself nor to a decrease in the sensitivity of the motor end-plates to acetylcholine. The chemical similarity of triethylcholine to choline, together with the fact that choline reverses the effects of triethylcholine, make it likely that triethylcholine interferes with the metabolism of choline at the nerve endings, probably by interfering with the synthesis of acetylcholine. The slow onset of the paralysis and its dependence on high activity in the motor nerves support this view, since the pre-formed depots of acetylcholine must be exhausted before transmission fails. Further work is necessary to elucidate the precise mechanism of action by which triethylcholine may interfere with acetylcholine synthesis, but it is of interest to speculate. The substance appears to resemble hemicholinium in its mechanism of action. (For a recent review of the actions of the hemicholiniums, see Schueler, 1960.) Hemicholinium has been found to prevent the synthesis of acetylcholine by the choline-acetylase of organized brain tissue but to be without this effect after disruption of the cell membranes (MacIntosh, Birks & Sastry, 1956; Gardiner, 1957). These results led to the suggestion that hemicholinium competes with choline for a carrier mechanism in the cell membrane and causes transmission failure at cholinergic junctions by rendering the nerve terminals deficient in choline and consequently in acetylcholine (MacIntosh, 1959). Triethylcholine and hemicholinium possess properties in common; both are more effective during high rates of nerve stimulation and both are antagonized by choline. The molecule of triethylcholine is small compared to that of the bisquaternary hemicholinium, and it is possible that, in addition to preventing the combination of choline with the carrier mechanism, triethylcholine may itself be transported to intracellular sites in place of choline. Burgen, Burke & Desbarats-Schonbaum (1956) found from *in vitro* studies that triethylcholine was acetylated by the choline acetylase of brain tissue almost as effectively as choline itself. The acetyl ester of triethylcholine is 5,000 times less active than acetylcholine on the frog rectus abdominis muscle (Holton & Ing, 1949), so that, if triethylcholine were acetylated in the nerve terminals and subsequently released as a "false" transmitter, it would be ineffective in causing muscle contraction.

The idea that triethylcholine might be synthesized into a physiologically inert neurohormone and thus cause transmission failure is not new. Burgen *et al.* (1956) and Stovner (1958) speculated on this possibility. The latter author, however, was unable to demonstrate such an effect in the isolated diaphragm preparation of the rat, probably because he used an insufficiently high rate of nerve stimulation.

Choline antagonizes tubocurarine block (Hutter, 1952), but this antagonism is short lasting in comparison with its reversal of triethylcholine block. Injected choline disappears rapidly from the circulation (Appleton, Levy, Steele & Brodie, 1951). Bligh (1953) found that the plasma choline in dogs returned to its normal level 10 to 20 min after intravenous injection of 5 mg/kg choline chloride. The main loss appeared to be due to destruction by choline oxidase in liver and kidney.

The property of choline to antagonize the actions of triethylcholine at the neuromuscular junction indicates that the level of choline normally present may modify the effects of triethylcholine. Schueler (1960) suggested that the variation in the toxicity of hemicholinium between different batches of mice might depend on the dietary choline intake. Bligh (1952) found that the mean free choline content of cat plasma was 0.7 μg/ml. (range 0.5 to 0.8); rabbit plasma had a higher choline content (mean 2.9 μg/ml., range 1.2 to 5.2). In our experiments we found that it was more difficult to produce neuromuscular block with triethylcholine in the rabbit than in the cat. It is conceivable that this difference is related to the different choline contents. According to Bligh (1952) human plasma contains 1 to 2 μg/ml. of choline.

In the cat, the soleus is a slow-contracting muscle containing a high proportion of red fibres; its function is concerned mainly with the maintenance of posture and as such it is adapted for prolonged activity. The tibialis anticus muscle, on the other hand, is a fast-contracting flexor muscle containing a high proportion of white fibres. Of the two muscles, the soleus was considerably more resistant to the paralysing action of triethylcholine and hemicholinium when maximal twitches of both were elicited at the same frequency. This difference in sensitivity suggests that the nerve terminals in the soleus muscle may contain greater reserves of preformed transmitter and provides a possible explanation of the surprising resistance of the respiratory muscles to the blocking action of triethylcholine. When tetanic contractions of the tibialis and soleus muscles were recorded the difference in sensitivity to triethylcholine was less marked. Denny-Brown (1929) showed that postural tone in the soleus muscle was maintained by rates of firing of the order of 10/sec. In the present experiments, the frequency of tetanic stimulation used was 50 or 100/sec and, therefore, well above the physiological range for this muscle. During such tetanic contractions the acetylcholine reserves are probably rapidly exhausted, and the tension produced will therefore depend upon the amount of acetylcholine which can be freshly synthesized in the presence of triethylcholine or of hemicholinium.

Unlike most drugs, the action of triethylcholine is greater after subcutaneous, intraperitoneal or intramuscular injection compared with intravenous injection. For example, in the conscious cat, rabbit and dog the effect of a dose given intramuscularly or intraperitoneally is about the same as twice the dose given intravenously. The effect of triethylcholine in producing muscular weakness is slow in onset and therefore is favoured by an injection route which results in a longer persistence in the circulation than would follow intravenous injection. Triethylcholine is not attacked by choline oxidase (Wells, 1954), but it is probably excreted rapidly by the kidney.

Desmedt (1958) has demonstrated the similarity between electromyograms of muscles from myasthenic patients and from cats injected with hemicholinium ; those of cats injected with tubocurarine were quite different in his test procedure. He suggested therefore that " the myasthenic condition results from a presynaptic biochemical defect chronically impairing synthesis of acetylcholine." In our experiments on conscious animals triethylcholine produced a state resembling myasthenia gravis, and these results lend some support to Desmedt's conclusions that there is a presynaptic biochemical defect in this disease. However, the defect in myasthenia gravis and that produced by triethylcholine differ in that the weakness of myasthenia is antagonized by anticholinesterases, while the effects of triethylcholine are not influenced. There are two possible explanations for the inability of anticholinesterases to antagonize triethylcholine. Firstly, triethylcholine itself may possess anticholinesterase activity. Secondly, if triethylcholine is acetylated in the nerve ending, the false transmitter released will be preserved by anticholinesterase (acetyl triethylcholine is hydrolysed by cholinesterase, Holton & Ing, 1949 ; Burgen *et al.*, 1956).

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STRUCTURE-ACTIVITY RELATIONSHIPS IN A SERIES OF 6-THIOXANTHINES WITH BRONCHODILATOR AND CORONARY DILATOR PROPERTIES

BY

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The bronchodilator activity of 47 compounds on the isolated guinea-pig tracheal ring preparation and the coronary dilator activity on the dog heart–lung preparation is described. 1,3-Disubstituted xanthines and 6-thioxanthines (theophyllines and 6-thiotheophyllines) were more active as bronchodilators and as coronary dilators than were 3,7-disubstituted 6-thioxanthines (6-thiotheobromines) or 1,3,7-trisubstituted 6-thioxanthines (6-thiocaffeines). None of the 6-thioxanthines was of any interest as a diuretic and none of the 6-thiocaffeines was of any interest as a central stimulant. Structure-activity requirements for potent bronchodilator and coronary dilator activity are discussed.

The potent bronchodilator and coronary dilator properties of the choline salt of 3-isobutyl-1-methyl-6-thioxanthine (M&B 5924) have already been described (Armitage & Wooldridge, 1960 ; Armitage, Boswood & Large, 1961). In the present paper, the *in vitro* bronchodilator activity of 1,3- and 3,7-disubstituted and 1,3,7-trisubstituted 6-thioxanthines on the guinea-pig tracheal ring preparation and the coronary dilator activity on the dog heart–lung preparation are reported. For brevity these three classes of compounds will be referred to as 6-thiotheophyllines, 6-thiotheobromines and 6-thiocaffeines respectively. Results on various theophyllines, that is, non-thio-analogues, are also included. In the light of these results, structure-activity relationships among 6-thioxanthines are discussed. The synthesis and chemical properties of the compounds discussed in this paper will be published elsewhere (Slack & Wooldridge).

METHODS

Bronchodilator and coronary dilator activity. The methods used for estimating bronchodilator activity on the isolated guinea-pig tracheal ring preparation and coronary dilator activity on the dog heart–lung preparation were fully described by Armitage *et al.* (1961). The tracheal chain preparation was found to work much more satisfactorily if the cartilage of each ring in the chain was cut, as described by Akçasu (1959).

Other methods used in the present study and described in our earlier paper include the estimation of *in vivo* bronchodilator activity, which was assessed by determining whether the

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compounds when given intraperitoneally or orally would protect guinea-pigs against broncho-constrictor aerosols. Several of the compounds were tested for diuretic activity in conscious rats and for their effects on the voluntary motor activity of mice by the light-box method of Dews (1953). Intravenous and oral LD50 values in albino mice were also determined.

Emetic properties of some of the more active 6-thiotheophyllines and theophyllines were studied in cats and dogs.

Effects on respiration of anaesthetized rabbits. The method used was that described by Burn (1952). Rabbits of either sex were anaesthetized with urethane and respiratory movements were recorded with a kymograph. Respiration was usually depressed by intravenous injection of 4 mg morphine sulphate. In some of the experiments, blood pressure was stabilized as described by Burn & Rand (1960).

RESULTS

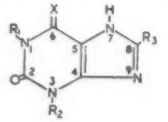
6-Thiotheophyllines

Bronchodilator and coronary dilator activity of 6-thiotheophyllines. The structures of the 6-thiotheophyllines tested are shown in Table 1 and their molar activity ratios in Table 2. These figures have been calculated as the ratio of the molar dose or molar concentration of choline theophyllinate required to cause a given relaxation of the guinea-pig tracheal muscle or a given increase in coronary blood flow in the dog heart–lung preparation, to the molar dose of 6-thiotheophylline required to produce a similar effect. It should be stressed that these estimates of activity, particularly coronary dilator activity, were for many of the compounds only approximate, the main object of the experiments being to ascertain which compound was the most active. The number of experiments was often small and the data have not been subjected to any statistical tests. Included for each compound in Table 2 are the mean activity ratio, the number of experiments and the highest and lowest activity observed in all the experiments. Activities have been calculated to the nearest whole number. Compounds were not always compared directly with choline theophyllinate but with a compound whose activity relative to choline theophyllinate had been determined in previous experiments. It will be seen that compounds 6, 19, 21 and 23 were the most active bronchodilators and compound 6 was the most active coronary dilator. In the dog heart–lung preparation, compound 23 caused predominantly coronary constriction as shown in Fig. 1.

The *in vivo* bronchodilator activity of 6 compounds (1, 2, 5, 15, 25 and 27) was investigated on 8 guinea-pigs for each compound, the animals being exposed 20 min after intraperitoneal injection to either a 2% histamine or 4% acetylcholine aerosol. Activity relative to choline theophyllinate was invariably less than on the guinea-pig tracheal ring preparation, compounds 2, 5 and 15 being only 1 to 2 times as active as choline theophyllinate, compounds 1 and 27 being equiactive and compound 25 being inactive and causing convulsions in a dose of 100 mg/kg salt. Experiments were also performed with compounds 1a, 5a, 6a, 19, 19a and 21 to see if, when given orally, they subsequently protected guinea-pigs from the convulsant effects of a 4% acetylcholine aerosol and for approximately how long the protection lasted. Choline theophyllinate and compound 21 were the least effective, 5a was about 3 times as active, and 19 and 19a were both about twice as active as choline theophyllinate. Activity was estimated as the ratio of the doses

TABLE 1

CHEMICAL STRUCTURES AND TOXICITY VALUES OF THEOPHYLLINES AND 6-THIOTHEOPHYLLINES



Compound no.	M&B no.	R_1	R_2	R_3	X	Salt	Mol. wt.	Oral LD50/ intravenous LD50 (mg/kg active component in mice)
1 (Choline 6-thiotheophyllinate)	4926A	CH_3–	CH_3–	H–	S	Choline	299	260/39=7
1a (Choline theophyllinate)	—	CH_3–	CH_3–	H–	O	Choline	283	337/74=4
1b (Aminophylline)	—	CH_3–	CH_3–	H–	O	Ethylenediamine dihydrate	456/2	250/150=1–2
2	5656	CH_3–	C_2H_5–	H–	S	Choline	313	244/47=5
3	6020	CH_3–	n–C_3H_7–	H–	S	Choline	327	250/63=4
4	6138	CH_3–	CH_2:CH.CH_2–	H–	S	Choline	325	260/48=5
5	5777	CH_3–	n–C_4H_9–	H–	S	Choline	341	364/55=7
5a	6052	CH_3–	n–C_4H_9–	H–	O	Choline	325	129/66=2
6	5924	CH_3–	i-C_4H_9–	H–	S	Choline	341	163/51=3
6a	6139	CH_3–	i-C_4H_9–	H–	O	Sodium	244	75/42=1–2
7	6375	CH_3–	CH_2:C(CH_3).CH_2–	H–	S	Choline	339	142/47=3
8	5955	CH_3–	n–C_5H_{11}–	H–	S	Choline	355	294/58=5
8a	6986	CH_3–	n–C_5H_{11}–	H–	O	Sodium	258	77/54=1–2
9	6340	CH_3–	$CH_3O.CH_2.CH_2.CH_2$–	H–	S	Sodium	276	345/132=3
9a	6847	CH_3–	$CH_3O.CH_2.CH_2.CH_2$–	H–	O	Base	238	210/130=1–2
10	6082	CH_3–	i–C_5H_{11}–	H–	S	Choline	355	425/51=8
11	5937	CH_3–	n–C_6H_{13}–	H–	S	Choline	369	>430/34=>12
12	6051	CH_3–	$C_6H_5.CH_2$–	H–	S	Choline	375	360/51=7
13	6522	CH_3–	$C_6H_5.CH_2.CH_2$–	H–	S	Choline	389	955/46=>20
14	6557	CH_3–	Furfuryl	H–	S	Sodium	284	460/135=3
14a	6761	CH_3–	Furfuryl	H–	O	Sodium	268	420/96=4
15	5722	C_2H_5–	CH_3–	H–	S	Choline	313	234/63=4
16	5861	C_2H_5–	C_2H_5–	H–	S	Choline	327	503/54=9
17	6511	C_2H_5–	CH_2:CH.CH_2–	H–	S	Sodium	258	450/110=4
18	5904	C_2H_5–	n–C_4H_9–	H–	S	Choline	355	510/68=7
19	6783	C_2H_5–	i–C_4H_9–	H–	S	Sodium	274	405/138=3
19a	6775	C_2H_5–	i–C_4H_9–	H–	O	Sodium	258	150/125=1–2
20	6510	CH_2:CH.CH_2–	C_6H_5–	H–	S	Sodium	306	1,450/130=11
21	6154	n–C_3H_7–	n–C_3H_7–	H–	S	Choline	355	>565/46=>12
22	5962	n–C_4H_9–	CH_3–	H–	S	Choline	341	>550/54=>10
22a	6985	n–C_4H_9–	CH_3–	H–	O	Sodium	244	235/140=1–2
23	6164	n–C_4H_9–	n–C_4H_9–	H–	S	Sodium	302	>500/21=>24
24	5829	CH_3–	CH_3–	CH_3–	S	Choline	313	193/66=3
25	5609	CH_3–	CH_3–	C_2H_5–	S	Choline	327	109/45=2
25a	6189	CH_3–	CH_3–	C_2H_5–	O	Sodium	230	180/130=1–2
26	5812	CH_3–	CH_3–	HS–	S	Choline	331	>2,000/69=>29

TABLE 2

STRUCTURE-ACTIVITY OF 6-THIOTHEOPHYLLINES, BRONCHODILATOR ACTIVITY (BDA), CORONARY DILATOR ACTIVITY (CDA), EXPRESSED RELATIVE TO CHOLINE THEOPHYLLINATE

Compound no.	Mean relative molar BDA	Range of BDA and no. of expts. in parentheses	Mean relative molar CDA	Range of CDA and no. of expts. in parentheses	Thioxanthine/xanthine activity BDA	Thioxanthine/xanthine activity CDA
1	5	3–7 (5)	3	2–5 (6)	5	3
1a	1	1 by definition	1	1 by definition		
1b	1–2	1–2 (6)	1–2	1–2 (2)	3	2
2	20	8–25 (7)	5	3–6 (8)		
3	44	39–60 (3)	7	— (1)		
4	13	10–15 (5)	7	3–10 (7)		
5	53	23–108 (17)	10	5–15 (11)	0·4	0·4
5a	134	75–214 (10)	22	12–37 (7)		
6	72	24–122 (15)	18	10–29 (14)	0·7	0·9
6a	102	54–183 (9)	20	20 (3)		
7	36	24–44 (3)	12	11–14 (3)		
8	59	44–83 (5)	10	7–14 (4)	0·5	0·4
8a	110	46–174 (3)	25	16–32 (3)		
9	20	11–28 (3)	7	7 (3)	2	2
9a	11	7–15 (2)	3	— (1)		
10	54	29–83 (3)	5	4–8 (3)		
11	23	23 (2)	4	3–5 (2)		
12	29	24–35 (3)	1	— (1)		
13	22	13–30 (3)	4	4–5 (3)		
14	10	— (1)	7	7 (2)	0·6	0·6
14a	16	16 (2)	12	4–16 (3)		
15	9	2–21 (7)	3	3 (3)		
16	36	32–41 (3)	5	4–5 (2)		
17	18	13–26 (4)	4	4 (3)		
18	69	42–93 (4)	4	— (1)		
19	143	102–208 (5)	6	6 (3)	0·4	0·7
19a	342	246–407 (5)	9	4–14 (5)		
20	49	37–53 (3)	<1	— (1)		
21	143	72–254 (5)	5	2–8 (6)		
22	4	3–6 (2)	2	— (1)	0·1	0·5
22a	32	— (1)	4	— (1)		
23	202	94–290 (4)	Constrictor	— (2)		
24	5	5 (2)	3	2–4 (4)		
25	11	7–24 (5)	4	2–7 (8)	2	2
25a	5	3–8 (3)	2	1–3 (4)		
26	Constrictor	— (1)	Not tested	—		

giving about equal protection of between 50 and 100%. Compound 6a was toxic to guinea-pigs in doses as low as 20 mg/kg, this dose killing 6 out of 32 guinea-pigs. At 10 mg/kg, compound 6 offered less than 50% protection, but as much protection as choline theophyllinate at 100 mg/kg. None of the compounds tested for *in vivo* bronchodilator activity was found to have more than slight antihistamine or anti-acetylcholine activity on the guinea-pig ileum.

Other properties of 6-thiotheophyllines. The pharmacological properties of M&B 5924 (compound 6) have already been described in detail (Armitage *et al.*, 1961). Further experiments have been made on some other 6-thioxanthines, and in general the only differences observed have been quantitative.

Diuretic activity in rats. These experiments were of a qualitative nature only. Rats were given by stomach tube 25 ml./kg 0.9% saline containing the appropriate

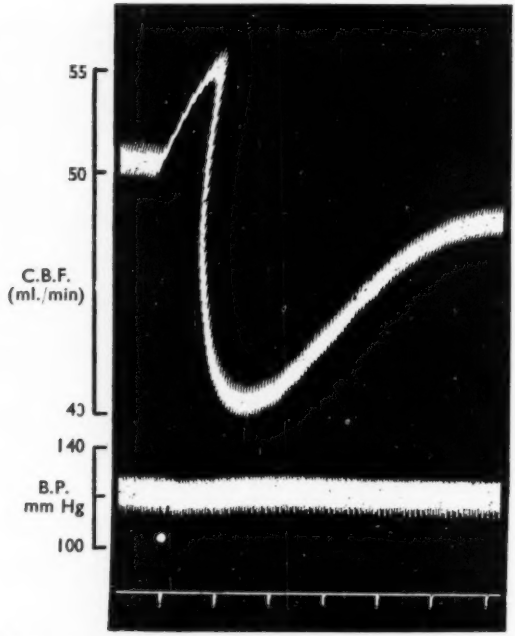


Fig. 1. Dog heart–lung preparation, showing coronary blood flow (upper record) and blood pressure (lower record). Time marker=60 sec. At the white dot, 0.5 mg compound 23 was injected close to the superior vena cava. Note the unusual effect in this series of compounds of coronary constriction following a small initial dilatation.

amount of compound to give doses up to 40 mg/kg. Urine was collected for 5 hr. Compounds 1, 2, 3, 5, 5a, 6, 8, 10, 11, 15, 18, 22 and 25 were all much less active than choline theophyllinate and were therefore of no interest as diuretics.

Effects on the voluntary motor activity of mice. In doses up to 20 mg/kg choline theophyllinate increased the motor activity of mice, a 50% increase being produced by 12 mg/kg theophylline. All the other theophyllines and 6-thiotheophyllines, however, in doses up to 80 mg/kg either decreased motor activity or had no effect, the dose causing a 50% decrease usually being in the range 20 to 50 mg/kg.

Emetic properties in cats and dogs. The results of the experiments on two of the most active pairs of theophyllines and 6-thiotheophyllines are shown in Table 3. The drugs were administered by injection into the saphenous vein and orally in gelatin capsules. Vomiting and retching sometimes occurred within a minute of intravenous injection, but recovery was usually complete within 1 or 2 hr. In cats, but not in dogs, salivation and laboured respiration were sometimes observed. Compound 19a caused emesis in cats and dogs in much lower doses than the other compounds. Compounds 4, 5, 5a and 7 were also given orally to dogs, and all caused vomiting at much lower doses than choline theophyllinate and aminophylline.

TABLE 3

EMETIC PROPERTIES OF THEOPHYLLINES AND 6-THIOTHEOPHYLLINES IN CATS AND DOGS

Compound no.	Dose (mg/kg active component)	Route	No. of dogs which vomited	No. of cats which vomited
6	2	Intravenous	2/5	—
(M&B 5924)	4	Intravenous	—	0/2
	5	Oral	0/2	—
	8	Intravenous	—	0/2
	10	Oral	2 2	0/2
6a	1	Intravenous	0/2	—
(M&B 6139)	2	Intravenous	2/5	0/2
	4	Intravenous	2/3	2/2
	5	Oral	2/2	—
	10	Oral	2/2	0/2
19	2	Intravenous	0/4	0/1
(M&B 6783)	4	Intravenous	4/4	0/1
	6	Intravenous	—	0/1
	10	Oral	0/1	0/1
	20	Oral	—	0/1
19a	0·25	Intravenous	1/1	—
(M&B 6775)	0·5	Intravenous	7/7	—
	2	Oral	2/2	2/2
	5	Oral	4/4	2/2

Acute toxicity in mice. Approximate acute intravenous and oral LD50 values were determined for each compound with up to 30 mice for each determination. The ratio of the two LD50 determinations is shown in Table 1, and for most of the compounds the value was below 6. A low value for this ratio is generally taken to indicate that oral absorption is good.

2-Thiotheophyllines

One compound only of this series was tested, namely, choline 2-thiotheophyllinate, and it was less active as a bronchodilator and as a coronary dilator than the 6-thio-analogue.

6-Thiotheobromines

The chemical structures of the compounds tested, other than theophyllines and 6-thiotheophyllines, are shown in Table 4. Three compounds only of this series were tested, and, although slightly more active than choline theophyllinate as coronary dilators and as bronchodilators, they were much less active than the closely related 1,3-dialkyl-6-thiotheophyllines (Table 5). Choline 6-thiotheobrominate (compound 27), like choline 6-thiotheophyllinate (compound 1), was a relatively poor diuretic. Toxicity data are also shown in Table 5.

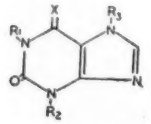
6-Thiocaffeines

The 6-thiocaffeines were tested for bronchodilator and coronary dilator activity and also compared with caffeine as central stimulants in the mouse motor activity test and for ability to stimulate the respiration of the anaesthetized rabbit depressed by morphine.

TABLE 4

CHEMICAL STRUCTURES OF 6-THIOTHEOBROMINES AND 6-THIOCAFFEINES

In column 8, Sal=dissolved in 25% sodium salicylate solution, Sal'=an equimolecular mixture of caffeine and sodium salicylate dissolved in water



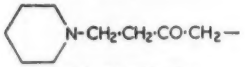
Compound no.	M&B no.	R_1	R_2	R_3	X	Salt	Solvent	Mol. wt.
27	4390A	H–	CH_3–	CH_3–	S	Choline	H_2O	299
28	6436	H–	n-C_4H_9–	CH_3–	S	Sodium	H_2O	260
29	6266	H–	i-C_4H_9–	CH_3–	S	Sodium	H_2O	260
30	6287	H–	i-C_4H_9–	H–	S	Sodium	H_2O	246
31	6227	CH_3–	CH_3–	CH_3–	S	—	Sal	210
31a	—	CH_3–	CH_3–	CH_3–	O	Citrate	H_2O	386
31b	—	CH_3–	CH_3–	CH_3–	O	—	Sal'	194
32	6231	CH_3–	CH_3–	C_2H_5–	S	—	Sal	224
33	6194	CH_3–	CH_3–	$(C_2H_5)_2N.CH_2.CH_2$–	S	Hydrochloride	H_2O	331
34	6316	CH_3–	CH_3–	$(C_2H_5)_2N.CH_2.CH_2$–	S	Methiodide	H_2O	437
35	6254	CH_3–	CH_3–	$CH_3.CO.CH_2$–	S	—	Sal	252
36	6496	CH_3–	CH_3–		S	Hydrochloride	H_2O	385
37	6464	CH_3–	i-C_4H_9–	$(C_2H_5)_2.N.CH_2.CH_2$–	S	(−)-Di(*p*-toluoyl)-D-tartrate	H_2O	723

TABLE 5

STRUCTURE-ACTIVITY OF 6-THIOTHEOBROMINES AND 6-THIOCAFFEINES, BRONCHODILATOR ACTIVITY (BDA), AND CORONARY DILATOR ACTIVITY (CDA), EXPRESSED RELATIVE TO CHOLINE THEOPHYLLINATE

Compound no.	Mean relative molar BDA and no. of expts. in parentheses	Mean relative molar CDA and no. of expts. in parentheses	Oral LD50/intravenous LD50 (mg/kg active component in mice)	Respiration
27	2–3 (1)	2 (4)	340/40=8	Not tested
28	16 (1)	1–2 (1)	>400/30=>13	Not tested
29	12 (3)	2 (1)	560/50=11	Not tested
30	19 (2)	3 (4)	177/79=2	Not tested
31	<1 (1)	1 (1)	960/—	+
31a	0·5 (2)	0·5 (2)	356/49=7	++
31b			312/—	
32	<1 (1)	2 (1)	515/—	+
33	5 (3)	1 (3)	228/19=12	++
34	Constrictor (1)	≪1 (1)	>420/14=>30	+
35	<1 (1)	1 (1)	—/—	+
36	2 (1)	1 (1)	472/34=14	++
37	<1 (1)	Not tested	>1,000/—	Not tested
Nikethamide	Not tested	Not tested	Not determined	+++

Bronchodilator and coronary dilator activity. Three of the 6-thiocaffeines (Table 4, compounds 31, 32 and 35) were soluble only in 25% (w/v) sodium salicylate solution, and some of the bronchodilatation and coronary dilatation caused by solutions of these compounds was due to the sodium salicylate content. Nevertheless, the bronchodilator activity of these compounds was low ; as coronary dilators they were more active than caffeine, but very much less active than the 6-thiotheophyllines.

Motor activity in mice. Caffeine citrate in doses of 20 to 40 mg/kg increased the voluntary motor activity of mice up to a maximum of 40% above the control count. None of the 6-thiocaffeines tested had any stimulant effects on mice, although high doses (80 mg/kg) sometimes caused sedation.

Effects on respiration. It was not easy to compare quantitatively the stimulant effects of 6-thiocaffeines, and for this reason the results shown in Table 5 are expressed merely in a qualitative way. For example, a dose of compound which at the beginning of an experiment caused perhaps 30% stimulation of the rate was sometimes without effect later in the experiment. The degree of stimulation varied according to the initial rate of respiration, the level of anaesthesia and blood pressure, in experiments in which blood pressure was not stabilized.

All the compounds were tested except 37. Compounds 31, 32 and 35 all caused stimulation of the respiration, most of which was shown to be due to the sodium salicylate solvent. Of the other compounds, there appeared to be little difference in the stimulant properties of 33, 36 and caffeine (31a, 31b). Some of these stimulant effects are shown in Fig. 2. Nikethamide and caffeine caused similar stimulation whether the blood pressure was stabilized or not, but the 6-thiocaffeines, which caused large falls in blood pressure in the normal preparation, were usually less effective stimulants when the blood pressure was stabilized. Compound 34 was

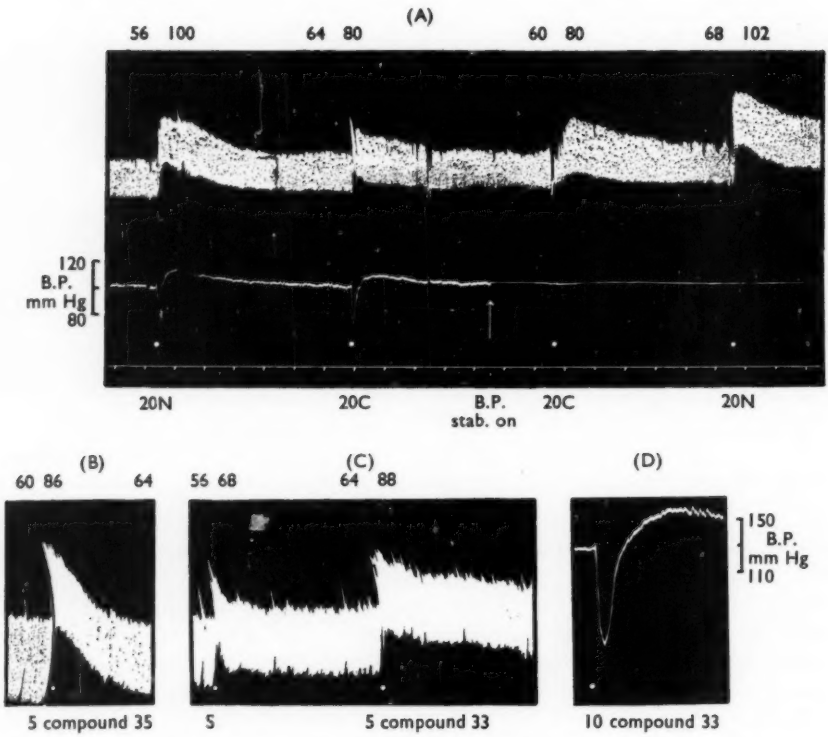


Fig. 2. Rabbits, urethane anaesthesia. Records of respiration and blood pressure in (A), respiration in (B) and (C) and blood pressure in (D). Respiration rates are shown at the top of each tracing. C=caffeine citrate, N=nikethamide, S=0·4 ml. sodium salicylate solution (25% w/v). Time marker in (A)=60 sec, also the same for (B), (C), and (D). The values with C, N, compound 35 and compound 33 refer to doses in mg/kg injected intravenously. In (A) are shown the effects of caffeine citrate and nikethamide on respiration before and after stabilizing the blood pressure. In contrast to the 6-thiocaffeines, caffeine citrate and nikethamide caused only small changes in blood pressure and blood pressure stabilization did not affect the respiratory response. In another experiment shown in (B) and (C), in which the blood pressure was stabilized at 100 mm Hg, compounds 35 and 33 had similar effects on respiration, part of which in the case of compound 35 was due to the high concentration of sodium salicylate (S). Time interval between (B) and (C)=35 min. In (D) is shown the typical effect on blood pressure of high doses of the 6-thiocaffeines, in this case compound 33, when the blood pressure was not stabilized.

less active than compound 33. All these compounds were active in the range 5 to 10 mg/kg, although 10 mg/kg of 33 and 36 killed one rabbit each. The dose required to stimulate respiration was not much less than the dose which caused convulsions and a profound fall in blood pressure. Nikethamide, on the other hand, could safely be given in doses up to 30 mg/kg without any pronounced effects on the blood pressure.

DISCUSSION

As far as we are aware, most of the 6-thioxanthines described in this paper are new compounds, the only other reference to compounds of this type being a paper by Quimby, Aviado & Schmidt (1958) on the effects of xanthines and some unspecified 6-thioxanthines on the pulmonary circulation of the dog. The first compound we tested was choline 6-thiotheophyllinate, which was more than twice as active as choline theophyllinate as a bronchodilator and as a coronary dilator. This suggested that 6-thiotheophyllines might be more active than theophyllines, but this generalization is clearly incorrect, and with several higher members of the series the theophyllines were more active than the 6-thio-analogues (Table 2, column 6). Some compounds were tested as their sodium salts when it was not possible to prepare the choline salts, and activities have accordingly been quoted in Tables 2 and 4 on a molar basis. Le Roy & Speer (1940) found that there was a difference in the coronary dilator activity of different salts of certain xanthines ; in particular the sodium acetate salt of theobromine was five times as active as the sodium salt. Such a large discrepancy is difficult to understand. The only compound we have tested as two salts is theophylline itself ; aminophylline, the ethylenediamine salt, was consistently about 1.5 times as active as the choline salt. Of several mono-, di-, and tri-alkylxanthines reported by Le Roy & Speer (1940), none was more active than aminophylline as a coronary dilator. These experiments were made in the anaesthetized dog, and so differences in the activity of 1,3-diethyl- and 1,3-dibutyl-xanthine compared with our results are not surprising. The latter was reported to cause no effect on coronary flow in two out of three experiments, whereas in two experiments we found it to cause coronary constriction.

1,3-Dialkylxanthines are emetic (Kattus, Newman & Franklin, 1951), as we have also found, and it is probably because of their poor tolerance that these compounds have not found use in man. 1-Ethyl-3-isobutylxanthine (compound 19) caused emesis in cats and dogs, both orally and intravenously, even when administered in very low doses (Table 3). It would appear that with alkyl groups larger than methyl in the 1- and 3- positions activity increases at the expense of tolerance. Some of the newer 7-hydroxyalkyl- and 7-aminoalkyl-theophylline derivatives are less acutely toxic than theophylline, but are no more active and apparently no better tolerated than existing preparations (McColl, Parker & Ferguson, 1956 ; Jourdan & Faucon, 1958 ; Roth, Winbury & Govier, 1957). The fact that the theophyllines and thiotheophyllines caused vomiting when administered intravenously as well as when administered orally indicated that vomiting was not solely due to gastric irritation.

Although we do not wish to place too much emphasis on the relative activities quoted in Tables 2 and 4, our results clearly illustrate some of the factors essential for bronchodilator and coronary dilator activity in these series of compounds.

Goodman & Gilman (1955) state that theophylline is more active as a bronchodilator and as a coronary dilator than theobromine and that theobromine is more active than caffeine. The same relationship exists for the 6-thio-analogues, 6-thiotheophylline being more active than 6-thiotheobromine or 6-thiocaffeine. So far as 6-thiotheophyllines are concerned, maximum bronchodilator activity was achieved with a relatively large alkyl group in the 3-position. Among 1-methyl-6-thiotheo-

phyllines, the 3-isobutyl derivative was the most active compound tested, though the n-butyl, n-pentyl and isopentyl derivatives were not much less active. With larger or smaller groups than these, however, activity was considerably reduced. In this series of compounds, coronary dilator activity generally ran closely parallel with bronchodilator activity, and the 3-isobutyl derivative was again the most active compound. It is interesting to note that compounds with an unsaturated group in the 3-position (for example, allyl, methylallyl, compounds 4 and 7 respectively) or compounds with a substituted alkyl group (for example, methoxypropyl, compound 9) were less effective as bronchodilators than the equivalent saturated or non-substituted compounds, 3, 6 and 8 respectively. The coronary dilator properties of the pairs of compounds, however, were very similar. From spatial considerations, a furfuryl group is theoretically equivalent to a 4-atom chain, but 3-furfuryl-1-methyl-6-thioxanthine (compound 14) was much less active as a bronchodilator and slightly less active as a coronary dilator than the 3-n-butyl derivative (compound 5).

Among 1-ethyl-6-thiotheophyllines, the 3-isobutyl derivative was the most active compound tested, being more active than the 1-methyl homologue as a broncho-dilator, though less active as a coronary dilator. This relationship was observed with all the 1-ethyl-6-thiotheophyllines. Only 4 compounds were tested with groups larger than ethyl in the 1-position, and 1,3-di-n-propyl-6-thioxanthine (compound 21) and 1,3-di-n-butyl-6-thioxanthine (compound 23) were exceptionally active as bronchodilators. Compound 23 also had the interesting property of first dilating, then constricting, the coronary vessels of the dog heart–lung preparation, and, although some 6-thioxanthines were almost inactive as coronary dilators, this was the only compound tested that caused the opposite of the expected action. It is possible that higher members of the series might be pure coronary constrictors. The other 1-n-butyl derivative (compound 22) was the reverse analogue of the active compound 5 and was only feebly active, indicating the importance of a small alkyl group in the 1-position. It should be noted that compounds 20, 21, 22 and 23 all had a high oral : intravenous LD50 ratio in mice, indicating that their oral absorption was poor. Of the three 8-substituted 6-thiotheophyllines tested, none had more than moderate bronchodilator or coronary dilator activity.

From these results it appears that a 1-methyl group is essential for high coronary dilator activity. Bronchodilator activity, however, was increased in compounds with relatively large groups in both the 1- and 3-positions, though with large groups in the 1-position oral absorption was probably reduced. It is also concluded that unsubstituted alkyl groups in the 3-position are essential for high bronchodilator activity as measured on the guinea-pig tracheal ring preparation, but are of less importance for coronary dilator activity. No conclusions can be drawn concerning structure-activity requirements for diuretic activity, since all the compounds tested had similar low activity. With the advent of the chlorothiazide-like diuretics, theophylline itself has become almost obsolete and it seems doubtful if 6-thioxanthines will be of any interest as diuretics. Of the dialkylxanthines, we tested only compound 5a for diuretic activity ; it was half as active as choline theophyllinate and slightly more active than its thio-analogue. Several dialkylxanthines, in

particular 1,3-diethylxanthine, were reported to be more active than theophylline as diuretics (Kattus *et al.*, 1951).

The 6-thiotheobromines and 6-thiocaffeines were all of little interest as bronchodilators and coronary dilators. Transfer of the methyl group from the 1- to the 7-position in compounds 28 and 29 greatly reduced activity, and the introduction of extra groups in the 7-position (6-thiocaffeines) also reduced activity. It is clearly only the 1,3-dialkyl-6-thioxanthines which are potent bronchodilators and coronary dilators.

The 6-thiocaffeines in contrast to caffeine did not show any stimulant properties in increasing the voluntary motor activity of mice, and although they did stimulate the respiration of the anaesthetized rabbit they were less effective than caffeine and nikethamide. The observation that they were less effective stimulants when the blood pressure was stabilized indicates that part of the stimulation is reflex in origin. Nikethamide, however, even in high doses, did not cause any undesirable vascular effects.

Of all the 6-thioxanthines, only the 6-thiotheophyllines are of any potential therapeutic interest. They are highly active bronchodilators and coronary dilators on the pharmacological preparations used, and there is some evidence in cats and dogs that they have less effect in producing emesis than their oxygen analogues.

We are grateful to our colleagues, Dr R. Slack and Dr K. R. H. Wooldridge, who initiated and developed this series of xanthines and thioxanthines, and to G. T. Langston, M. A. Ogilvie and A. C. Rasmussen for excellent technical assistance with the dog heart–lung preparations.

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ON THE QUESTION OF THE OCCURRENCE AND METABOLISM OF 5-HYDROXYTRYPTAMINE AND RELATED INDOLE COMPOUNDS IN MAMMALIAN SEMEN

BY

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The content of 5-hydroxytryptamine was examined in the semen of man, bull, ram, boar and dog. Spectrophotofluorimetric and chromatographic analysis of extracts prepared by several methods has shown that the semen contains only minute quantities of 5-hydroxytryptamine, if any. One cannot attribute to 5-hydroxytryptamine the uterus-stimulating effect which the human seminal plasma is known to exert. Tryptophan, although present in the seminal plasma, is not converted to 5-hydroxytryptamine on incubation of semen. 5-Hydroxytryptophan is oxidatively deaminated, but not decarboxylated, by spermatozoa. 5-Hydroxytryptamine itself, like tryptamine and tyramine, is not deaminated by either whole semen or washed suspensions of ram spermatozoa.

Among the pharmacodynamic effects of the mammalian seminal plasma is a strong stimulation of smooth-muscle organs such as the uterus and the intestine. This oxytocic activity of semen was investigated in the past by many authors, including Kurzrok & Lieb (1930), Cockrill, Miller & Kurzrok (1935), Goldblatt (1933, 1935a, b), von Euler (1934a, b; 1935, 1936, 1939, 1949), Vandelli (1943), Asplund (1947a, b), Karlson (1949) and Bergström (1949); the general conclusion reached was that the uterine-stimulating property of semen, particularly in man, is probably due to the combined action of several constituents of the seminal plasma, such as choline, prostaglandin and histamine. The early literature has been reviewed in some detail by Mann (1954). Seminal prostaglandin has since been purified and is now believed to be produced, at least in man and ram, by the seminal vesicles and not by the prostate gland (Eliasson, 1959). Two crystalline compounds, both possessing the properties of nitrogen-free unsaturated fatty-acids, have been separated from prostaglandin preparations of vesicular origin; one of these, "prostaglandin E," was shown to exert, in addition to the smooth-muscle stimulating action, a depressant effect on blood pressure of the rabbit, while the other, "prostaglandin F," was found to be devoid of action on blood pressure (Bergström, Eliasson, Euler & Sjövall, 1959; Bergström, Duner, Euler, Pernow & Sjövall, 1959).

Several authors have also considered the possibility that the "oxytocic activity" of semen is due, at least partly, to 5-hydroxytryptamine, which is known to induce

strong uterine contractions, either when applied to the isolated uterus of the rat (Erspamer, 1952) or injected into a living dog (Abrahams & Pickford, 1956). The evidence, however, for the presence of this substance in mammalian semen has so far been confusing and contradictory. Thus, whereas according to Katsh (1959) human seminal plasma contains as much as 135 μg/ml., Hawker, Roberts & Walmsley (1960) could not find 5-hydroxytryptamine either in human or in ram semen.

On the other hand, a recent investigation of the secretory function of male accessory organs in the spiny dogfish, *Squalus acanthias*, has revealed the surprising fact that, in this viviparous elasmobranch fish, one of the accessory organs, the so-called clasper siphon, produces a secretion which is extremely rich in 5-hydroxytryptamine (Mann, 1960). The presence of as much as 7% of 5-hydroxytryptamine in a secretion which forms part of the seminal plasma in the spiny dogfish suggests that in this animal 5-hydroxytryptamine may be involved in the process of reproduction, either by facilitating the ejaculation of semen in the male or perhaps by eliciting contractions of the reproductive tract in the female and thus aiding sperm ascent in the oviducts. Another recent observation relevant to the function of 5-hydroxytryptamine in reproduction concerns certain insects. It was shown that the secretion of the opaque accessory gland in the male of *Rhodnius prolixus*, as well as the secretion of similar glands, the utriculi majores, in the cockroach *Periplaneta americana*, contain a substance which causes contractions of the insects' oviducts. The active principle is destroyed by monoamine oxidase and *o*-diphenol oxidase, and is probably an *o*-dihydroxyindolalkylamine related to 5-hydroxytryptamine (Davey, 1960).

We have attempted to clarify the question concerning the presence of 5-hydroxytryptamine and related compounds in mammalian semen, and to provide more information on the relation of 5-hydroxytryptamine to the "oxytocic activity" of semen.

METHODS

Semen. Semen from rams, bulls and boars was collected by means of the artificial vagina. Dog semen provided by Dr D. Bartlett was collected by the massage technique. Human semen from donors at the Fertility Clinic was made available by Dr H. A. Davidson. The semen samples intended for determination of 5-hydroxytryptamine were kept in solid carbon dioxide until deproteinized. In addition to whole boar semen, analyses of 5-hydroxytryptamine were also carried out on the seminal gel and the epididymal seminal plasma of this species. For measurements of respiration, fresh ram semen was used, either whole or after separation into spermatozoa and seminal plasma.

Spectrophotofluorimetric determination of 5-hydroxytryptamine. This was carried out in the Aminco-Bowman spectrophotofluorimeter on extracts prepared by one of the following three procedures: (1) The sample was deproteinized with zinc sulphate and sodium hydroxide; deproteinization was usually carried out by adding to the sample 1 vol. water, followed by 1 vol. of 10% zinc sulphate ($ZnSO_4.H_2O$) and 0.2 vol. of 10% sodium hydroxide; on occasions when the centrifuged extract was not completely clear, small additional amounts of the deproteinizing agents had to be added; to 2 ml. of the protein-free filtrate was added 1 ml. concentrated hydrochloric acid containing ascorbic acid (0.1 mg/1 ml.); the solution was placed in a quartz cuvette for spectrophotofluorimetric analysis. (2) The sample was extracted by the method of Bogdanski, Pletscher, Brodie & Udenfriend (1956). (3) The sample was treated with 4 vol. acetone and the resultant precipitate and supernatant solution processed further as described by Katsh (1959).

Chromatographic identification of 5-hydroxytryptamine and related indole compounds. Both descending and ascending one- and two-dimensional paper partition chromatography was used. The solvent systems, (i) butanol + acetic acid, (ii) isopropanol + ammonia, (iii) aqueous potassium chloride, and (iv) butanol + pyridine, were used as recommended by Jepson (1960). The developed chromatograms were dried at 25° C in an air oven, all fluorescent areas noted, and an ultra-violet contact photograph taken, using a Hanovia "Chromatolite" lamp and Kodak Duostat 13 reflex paper. The following location reagents, used either singly or in sequence, were applied: acid ninhydrin, ninhydrin pyridine, Ehrlich's reagent, diazotized sulphanilic acid (Jepson, 1960), 5 N hydrochloric acid (Sharman, 1960), and potassium dichromate formaldehyde (Shepherd & West, 1953).

Metabolic studies. Oxygen uptake was determined in Barcroft differential manometers filled with air, at 37° C. Ammonia formation was followed by treating the samples with a saturated solution of sodium borate and subjecting the mixtures to steam-distillation *in vacuo*, at a temperature not exceeding 25° C, in the apparatus of Parnas & Heller (1924). Monoamine oxidase activity was determined by measuring O_2 uptake and ammonia formation as well as by the colorimetric method of Green & Haughton (1961).

Chemicals. The chemicals were of the analytical or microanalytical reagent grade: 5-Hydroxytryptamine creatinine sulphate (Sigma Chem. Co.; May & Baker); dl-tryptophan (Light & Co.); 5-hydroxytryptophan (Roche); tryptamine and tyramine hydrochlorides (British Drug Houses).

RESULTS

Spectrophotofluorimetric observations

Experiment no. 1

This was carried out on a series of extracts prepared from human, ram, bull and boar semen by deproteinization with zinc hydroxide, and the supernatant then rendered 3 N with respect to hydrochloric acid. When these extracts were examined in the spectrophotofluorimeter using a Chance OY 4 filter in the fluorescent light path, a distinct fluorescence was observed with an activation maximum at 295 mμ and a fluorescence maximum at 525 mμ. This fluorescence maximum, however, was shown to be an artefact, due to the transmission characteristics of the filter, since, when the determinations were repeated with the filter omitted, the wavelengths for maximal activation and fluorescence of the seminal extracts were 295 mμ and 430 mμ, respectively. On the instrument used, 5-hydroxytryptamine, in common with other 5-hydroxyindolyl derivatives, showed a fluorescence in 3 N HCl, characterized by an activation maximum at 285 mμ and a fluorescence maximum at 540 mμ. Thus the seminal constituent which fluoresced with a maximum at 430 mμ could not be identical with 5-hydroxytryptamine, even though its activation peak of 295 mμ is close to that of 5-hydroxytryptamine.

In the light of this observation, subsequent fluorimetric analyses of the seminal extracts were made using an activation wavelength of 285 mμ and a fluorescence wavelength of 540 mμ, and calibrating each time the observed fluorescence against one produced by a standard solution of pure 5-hydroxytryptamine. When this was done the following results were obtained, the 5-hydroxytryptamine content being expressed in ng/2 ml. extract: three different samples of human semen, 0 to 5; ram semen, 0; ram seminal plasma, 0; two different samples of bull semen, 0 and 60; boar semen (separated from the gel), 250; boar seminal gel, 0; boar epididymal seminal plasma, 0.

As can be seen, the fluorescence values were of an exceedingly low order in all species. Moreover, these values represent not only the maximum fluorescence attributable to 5-hydroxytryptamine, but include, in all probability, the fluorescence due to some other substances which are present in semen.

Experiment no. 2

For the detection of trace amounts of 5-hydroxytryptamine in semen, an experiment was carried out on two 1.5 ml. samples of human semen, to one of which 5 μg 5-hydroxytryptamine was added before deproteinization. Both samples were extracted with butanol according to Bogdanski *et al.* (1956), and the purified and acidified extracts used to determine the activation scans with fluorescence at 540 mμ, as well as the fluorescence scans with activation at 280 mμ. The results of these determinations are recorded in Fig. 1, which shows the characteristic fluorescence

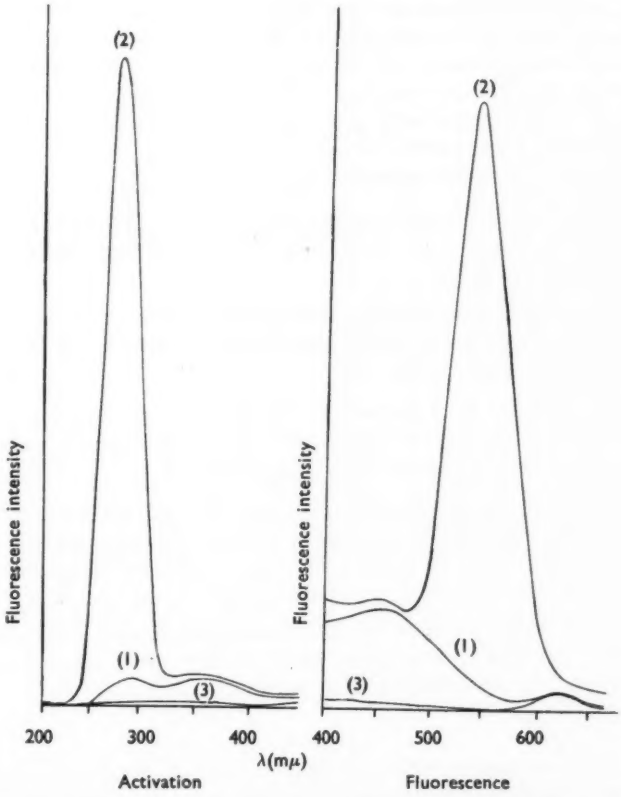

Fig. 1. Activation and fluorescence spectra of extracts from human semen: (1) extract from 1·5 ml. human semen; (2) extract from 1·5 ml. human semen to which 5 μg 5-hydroxytryptamine was added; and (3) reagent blank.

due to added 5-hydroxytryptamine and the lack of fluorescence in the sample without 5-hydroxytryptamine. These results clearly indicate that the content of 5-hydroxytryptamine in human semen is either negligible or nil.

Experiment no. 3

The fluorimetric analyses of human, bull, boar and ram semen were repeated, using larger quantities of semen and the extraction procedure of Bogdanski *et al.* (1956) which allows the preparation of more concentrated extracts. In addition, dog semen was examined by the same method. In each instance two measurements were taken: (i) of the wavelengths of maximal activation and fluorescence of the seminal extracts; and (ii) of the "5-hydroxytryptamine equivalent" of any fluorescence present, determined at the activation wavelength of 285 mμ, and the fluorescence wavelength of 540 mμ. The results were as follows.

Human semen. 9 ml. representing pooled ejaculates from four donors; two normal, one completely azoospermic, and one with sperm density less than 10,000 cells/μl.; final volume of the hydrochloric acid extract, 5 ml. When examined in the spectrophotofluorimeter, the extract exhibited a characteristically strong fluorescence with an activation maximum of 295 mμ and a fluorescence maximum at 430 mμ; 2 ml. of the hydrochloric acid extract, when examined at the 285 and 540 mμ wavelength, corresponded to a content of 540 ng "5-hydroxytryptamine equivalent," that is, 150 ng/ml. semen.

Bull semen. 13 ml. obtained from two bulls. The acid extract exhibited the typical 295/430 mμ fluorescence. The "5-hydroxytryptamine equivalent" content was 1,000 ng (1 μg)/ml. semen.

Ram semen. 15 ml. representing pooled ejaculates from ten rams. The acid extract exhibited the typical 295/430 mμ fluorescence. The "5-hydroxytryptamine equivalent" was 500 ng/ml. semen.

Boar semen. Out of a total ejaculate of 270 ml., a 15 ml. sample was used for extraction. The extract showed the typical 295/430 mμ fluorescence, and contained a "5-hydroxytryptamine equivalent" of 50 ng/ml. semen.

Dog semen. 15 ml. representing two ejaculates. The extract prepared from dog semen clearly showed the blue fluorescence previously observed in human, bull, ram and boar semen, namely, at the 295 and 430 mμ wavelengths. It contained a "5-hydroxytryptamine equivalent" of 150 ng/ml. semen.

The general conclusion to be drawn from experiment no. 3 is that there is very little 5-hydroxytryptamine, if any, in the semen of man, boar, ram, bull and dog. This was confirmed by the following experiment.

Experiment no. 4

Both the activation and fluorescence scans were determined using side by side the hydrochloric acid extracts obtained from human and canine semen, and a similarly prepared extract from the nucleus caudatus of the dog brain, of which the 5-hydroxytryptamine content had previously been determined and found to be

0.3 μg/g tissue. The results of this experiment are recorded in Fig. 2. In the left part of this figure the activation scan of the extract from dog semen is compared with that obtained from the nucleus caudatus. There is only a small difference in the maximum activation wavelength (295 mμ and 285 mμ, respectively). In the right part of Fig. 2, the fluorescence scan of human semen is contrasted with that of the dog nucleus caudatus; here it is obvious that the peak characteristic of 5-hydroxytryptamine (540 mμ) is entirely absent in the semen.

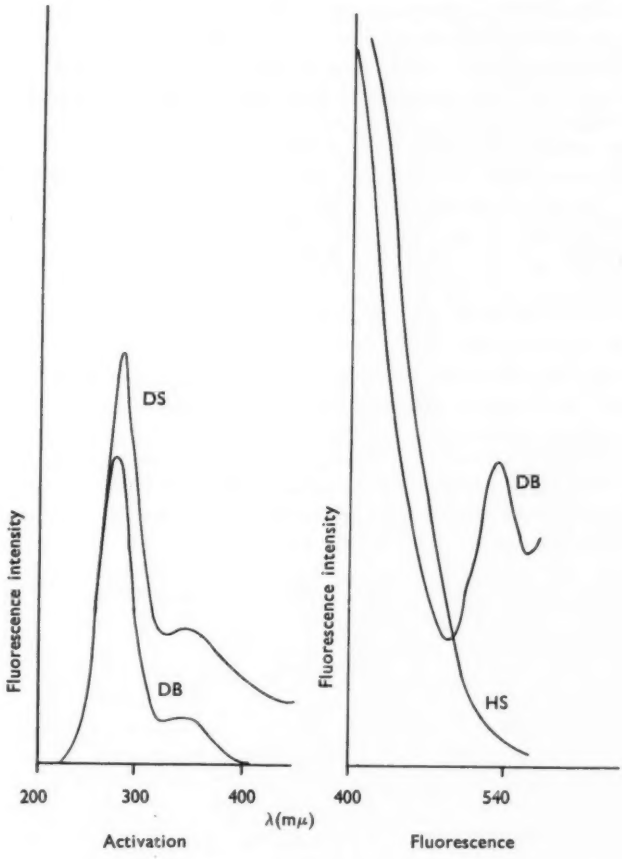

Fig. 2. Activation and fluorescence spectra of extracts from dog semen (DS), human semen (HS) and the nucleus caudatus of the dog brain (DB). The amplification used in recording the fluorescence scan of the extract from human semen (HS) was reduced to enable the record to cover the same wavelength range as that obtained with the nucleus caudatus extract (DB). If HS had contained 1 μg 5-hydroxytryptamine, a peak of the same size as that shown b y DP would have been observed.

Chromatographic observations on fresh semen

Several attempts were made to detect 5-hydroxytryptamine by chromatography in concentrated extracts from either whole semen or freshly prepared washed sperm suspensions and seminal plasma of man, bull, ram, boar and dog. The chromatograms were examined by the various methods already cited, but no evidence was obtained for the occurrence of 5-hydroxytryptamine.

In order to repeat the observation of Katsh on the presence of 5-hydroxytryptamine in human semen, two separate experiments were carried out, with 3 ml. and 12 ml. human seminal plasma, following closely the acetone extraction procedure. These experiments, too, failed to yield positive evidence for the presence of 5-hydroxytryptamine in human seminal plasma. It may be added that chromatograms developed with the various systems used revealed several substances reacting with one or another of the spraying reagents. However, no indole compounds other than tryptophan, no substituted tryptamine derivatives such as 5-hydroxytryptamine, nor any phenolic indole compounds were detected. Similarly, we obtained negative results with human semen using different deproteinization and extraction procedures (Udenfriend, Weissbach & Brodie, 1958).

We did not succeed in demonstrating 5-hydroxytryptamine chromatographically in bull, ram, boar and dog semen by any of the methods applied in this work.

Metabolism of 5-hydroxytryptamine and other indole compounds in semen

A series of chromatographic analyses was performed on ram semen, washed sperm and seminal plasma, which had been incubated in the presence of added 5-hydroxytryptamine or certain other related compounds, to find out whether 5-hydroxytryptamine itself is metabolized in semen, and also to explore the possibility that it might be formed in semen as the result of the metabolic breakdown of substances such as tryptophan and 5-hydroxytryptophan. Most of these experiments were combined with measurements of the oxygen uptake and ammonia evolution during the incubations.

5-Hydroxytryptamine. The experimental samples contained 5 mg 5-hydroxytryptamine creatinine sulphate dissolved in 1 ml. Ringer-phosphate, and 2 ml. of either a washed sperm suspension (10^9 cells) in Ringer or 2 ml. of correspondingly diluted whole semen; aerobic incubations were done in Barcroft manometers for periods ranging from 1 to 4 hr at 37° C. Under these conditions the addition of 5-hydroxytryptamine had no detectable effect on the rate of oxygen uptake, and there was no rise in free ammonia. After deproteinization with barium hydroxide and zinc sulphate {1.5 ml. incubation mixture + 0.5 ml. 0.3 N barium hydroxide + 0.5 ml. 5% zinc sulphate ($ZnSO_4.7H_2O$)}, 50 to 100 μl. aliquots of the protein-free filtrates were analysed by paper chromatography in various solvents. However, as a result of the aerobic incubation there was no evidence that either washed spermatozoa or whole semen produced any change in the 5-hydroxytryptamine content. In particular, there was no formation of 5-hydroxyindole acetic acid as an oxidative breakdown product of 5-hydroxytryptamine.

Tryptophan. The oxidative deamination of tryptophan by bull spermatozoa has been the subject of a study by Tosic & Walton (1950) and Tosic (1951), who have

pointed out that the formation of hydrogen peroxide during the oxidative deamination of aromatic amino-acids is responsible for the gradual decline in the oxygen uptake of incubated sperm mixtures. Using the same conditions as in the experiments with 5-hydroxytryptamine, we found that whole ram semen and washed ram spermatozoa can deaminate dl-tryptophan aerobically; moreover, whilst the same amount of ammonia was produced in the presence as in the absence of pure catalase, the rate of oxygen consumption was much higher with catalase present, owing to the decomposition of the toxic hydrogen peroxide. Chromatographic analyses of the incubation mixtures of dl-tryptophan and either whole semen or washed sperm suspensions showed that ram spermatozoa metabolize tryptophan aerobically, but not anaerobically, to several products. The major product was identified chromatographically as indoleacetic acid. The identification rested on the use of both descending and ascending chromatography in butanol + acetic acid (R_F 0.88 and 0.92, respectively), as well as in two other solvents, namely, isopropanol + ammonia (R_F 0.28) and potassium chloride (R_F 0.60). No tryptamine, 5-hydroxytryptophan or 5-hydroxytryptamine was detected.

5-Hydroxytryptophan. Mixtures of sperm and 5-hydroxytryptophan were incubated under the same conditions as in experiments with 5-hydroxytryptamine and tryptophan, in the presence and absence of catalase. A small amount of ammonia was formed, but there was no evidence of decarboxylation of 5-hydroxytryptophan to 5-hydroxytryptamine.

Tryptamine and tyramine. No ammonia formation occurred when a solution containing 5 mg tryptamine was incubated aerobically for 3 hr at 37° C, with either a suspension of washed spermatozoa (10^9 cells/2 ml. Ringer) or a sample of correspondingly diluted whole ram semen. When the experiment was repeated using tyramine instead of tryptamine, only a trace of ammonia was produced. These experiments provided a good indication that the monoamine oxidase activity of ram semen must be low. Supporting evidence for this view was obtained in the following manner. Tyramine oxidation was examined by the more sensitive procedure of Green & Haughton (1961), which depends on trapping with semicarbazide the aldehyde formed during the enzymic oxidation of tyramine, and converting the semicarbazone into the corresponding 2:4 dinitrophenylhydrazone. The light absorption of the orange-coloured alkaline solution of the dinitrophenylhydrazone was determined in the Unicam spectrophotometer at 450 mμ. Aerobic incubation for 2 hr at 37° C of a mixture (4 ml.) containing 1 ml. whole semen, 1 ml. 0.5 M semicarbazide solution (*p*H 7.4), 0.4 ml. 0.1 M solution of tyramine hydrochloride (*p*H 7.4), and 1.6 ml. Ringer phosphate produced less than 1/25 of the amount of semicarbazone which had been formed, under identical experimental conditions, by a preparation of mitochondria from 1 g of rat liver.

DISCUSSION

It was thought at one time that spermatozoa deposited at ejaculation in the vagina or cervix rely on their own motility in order to traverse the uterus and reach the oviducts. More recently, however, when measurements were made of the time interval required for some spermatozoa, at any rate, to arrive at the site of fertili-

zation, this proved to be remarkably short, in some species no more than a few minutes. This and subsequent work has shown that ejaculated spermatozoa are propelled to their final destination in the oviducts not so much by their own movements as by the concomitant uterine contractions.

The mechanism responsible for the increase in uterine motility following copulation and sexual stimulation is still under investigation. Some investigators attribute it mainly to the release of oxytocin by the pituitary gland ; others believe that semen itself may provide, at least partly, the required stimulus in the form of pharmacologically active constituents. Similarly, the chemical identity of the various " oxytocic " substances in semen is still under dispute, as shown by the perusal of recent literature (Mann, 1954 ; Eliasson, 1959 ; Hawker *et al.*, 1960).

The possibility that 5-hydroxytryptamine is one of these substances has been examined. The present study does not support the claim that mammalian semen has a high content of 5-hydroxytryptamine ; our spectrophotofluorimetric as well as chromatographic analyses have shown that the amine is either altogether absent from the semen of man, bull, boar, ram and dog, or present only in minute quantities. 5-Hydroxytryptamine may, perhaps, appear in human semen in measurable quantities in special circumstances. One need only recall the well-known effect which 5-hydroxytryptamine contained in certain foods exerts on the excretion of indole derivatives in the human urine. Certain other substances administered orally can pass unchanged into the seminal plasma ; for instance, when ergothioneine, a normal constituent of the seminal plasma, is fed to a boar the administered compound is found unchanged in the ejaculated semen (Heath, Rimington, Glover, Mann & Leone, 1953). The fact remains that 5-hydroxytryptamine is not a normal constituent of seminal plasma, either in man or in ram, bull, boar and dog. It is therefore unlikely that the uterine-stimulating property of semen depends upon the presence therein of 5-hydroxytryptamine.

An indole compound which is a normal constituent of mammalian seminal plasma is tryptophan. This amino-acid, however, and the closely related 5-hydroxytryptophan, do not appear to undergo any appreciable decarboxylation in semen. 5-Hydroxytryptamine itself, and related amines such as tryptamine and tyramine, when added to semen, are also relatively stable, as shown by incubation experiments carried out with whole ejaculated semen or washed sperm suspensions.

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SYNTHETIC OXYTOCIN AS AN ANTAGONIST OF EXPERIMENTAL CARDIAC ANOXIC CHANGES IN RABBITS

BY

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Synthetic oxytocin (Syntocinon) can be shown to reduce or abolish ST-T changes induced experimentally by hypoxaemia alone, by hypoxaemia and ergometrine, by vasopressin, and by a new procedure involving injection of small doses of picrotoxin into the lateral cerebral ventricle. Ventricular fibrillation induced by picrotoxin can also be reversed by oxytocin. These effects suggest a probable metabolic action of oxytocin against cardiac anoxic changes, and its possible therapeutic usefulness as an antagonist of myocardial ischaemia. It is speculated that this might be a physiological action of the hormone.

Rinzler, Travell, Karp & Charleson (1956) have reported that ergometrine (ergonovine) produces transient ST segment depression in rabbits with experimental coronary atherosclerosis. Stein (1949) has also employed these ST changes as a diagnostic procedure in patients with angina pectoris. In connexion with other studies from this laboratory (Varma & Melville, 1961a) it was observed that ergometrine intensified ST-T depression in the electrocardiogram associated with hypoxaemia in rabbits with experimental coronary atherosclerosis, but not in normal rabbits. Since ergometrine and oxytocin are both primarily uterine stimulants, it was of interest to test the effects of synthetic oxytocin (Syntocinon) upon the changes in the electrocardiogram induced by hypoxaemia. It was immediately observed that oxytocin strikingly antagonized these changes.

It had previously been reported (Melville, 1936) that, in unanaesthetized dogs, pressor pituitary preparations induced a fall in blood pressure which is abolished by simultaneous injections of large doses of oxytocic preparations. It is also well known that vasopressin induces experimentally coronary constriction and T-wave changes (Melville, 1939). It was therefore of interest also to investigate the effects of synthetic oxytocin upon these latter changes.

Finally, it has recently been observed (Share & Melville, 1961) that sustained ST-T depression (suggesting myocardial hypoxia) and various types of ventricular arrhythmias, including fibrillation, can be produced experimentally by injections of small doses of picrotoxin into the lateral cerebral ventricle of cats. While the exact mechanism of production of these cardiac changes is still uncertain, preliminary studies (Varma, Share & Melville, 1961) suggest that they might be due

to central sympathetic stimulation. As an additional method it was nevertheless of interest to study the effects of oxytocin upon the cardiovascular changes induced by this procedure.

METHODS

Albino rabbits (males and females, 2.5 to 3.5 kg) anaesthetized with intravenous pentobarbitone sodium (20 to 40 mg/kg) were used in these experiments. Coronary atherosclerosis was induced in male rabbits by prolonged (5 to 9 months) feeding with a diet containing 2% cholesterol and 6% corn oil in Purina Chow, as described by Melville & Shister (1959). Electrocardiograms (leads II and V) and femoral arterial blood pressure (using a Sanborn pressure transducer) were recorded concurrently. For the experiments with hypoxia the animals were placed in a large box (100 l. capacity) and a mixture of 10% oxygen and 90% nitrogen was blown through a flowmeter into the box at a rate of approximately 10 l./min, so that the air is completely displaced within 10 min. The effects of synthetic oxytocin (Syntocinon) were studied after both hypoxia (10 to 20 min) alone and hypoxia combined with an injection of 0.05 mg/kg of ergometrine maleate (Ergotrate maleate). In the experiments with vasopressin, Pitressin was used. After the cardiovascular responses to a dose of 1.0 i.u./kg of vasopressin were established, the injection was repeated 90 min later immediately following oxytocin, and a third similar injection of vasopressin alone tested after a further period of 90 min. This was necessary since the responses to repeated vasopressin were somewhat reduced even after 90 min. In the experiments with picrotoxin the effects of oxytocin were studied both during the period of ST-T depression in some experiments, and immediately following the occurrence of ventricular fibrillation in other experiments. All injections (except picrotoxin) were given intravenously through a polythene cannula attached to a needle inserted into the marginal ear vein of the rabbit.

RESULTS

Fig. 1 shows examples of the electrocardiogram and blood pressure changes recorded following injections of oxytocin, either during hypoxia alone (A) or during hypoxia combined with ergometrine (B). Similar results were observed in 10 atherosclerotic rabbits. As can be seen during either hypoxia alone or hypoxia with ergometrine, when marked ST-T depression was evident, intravenous injection

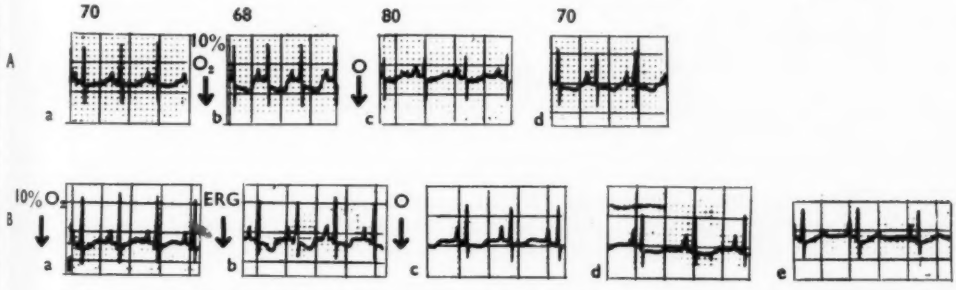

Fig. 1. Effects of oxytocin (1.0 i.u./kg intravenously) on electrocardiogram (lead V) changes induced by ergometrine and/or hypoxaemia in atherosclerotic rabbits under pentobarbitone sodium anaesthesia. A: Male 3.5 kg rabbit (fat-cholesterol diet for 8 months); a, control; b, after 10 min of hypoxia; oxytocin (O) injected after b and hypoxia continued; c, 1 min, and d, 10 min, after oxytocin. B: Male 3.5 kg rabbit (fat-cholesterol diet for 9 months); a, after 15 min of hypoxia; ergometrine (Erg.) 0.05 mg/kg injected after a; b, 3 min after ergometrine; oxytocin then injected; c, 10 sec, d, 1 min, and e, 2 min, after oxytocin.

of oxytocin (1.0 to 2.0 i.u./kg) led to prompt temporary reversal of the ST-T changes. This effect occurred within 30 sec, reaching its peak in 1 to 3 min, but with continued hypoxia ST-T depression gradually reappeared in 5 to 10 min. Oxytocin also induced a transient bradycardia and a variable slight rise in blood pressure.

In other experiments reported elsewhere (Varma & Melville, 1961b) it was observed that coronary dilator drugs, including glyceryl trinitrate, do not antagonize the ST-T changes induced by this type of hypoxaemia. Indeed, all the agents studied either exerted no effect or enhanced the ST-T depression observed. It would therefore appear that this antagonistic action of oxytocin is probably not due to coronary dilatation.

In the next series of experiments, the effects of oxytocin upon the cardiovascular changes induced by vasopressin were studied in 8 normal rabbits. Fig. 2 shows a typical example of the results obtained in all these experiments. Thus, after the

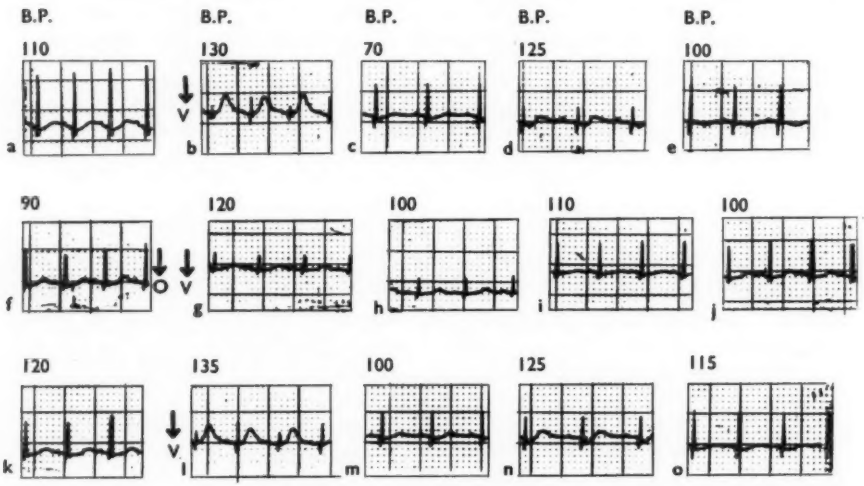

Fig. 2. Effect of oxytocin (5 i.u./kg intravenously) on the electrocardiogram (lead V) and mean arterial pressure (B.P. mm of Hg) changes induced by vasopressin (V)—1 i.u./kg—in a normal 3 kg male rabbit under pentobarbitone sodium anaesthesia. a, Control; vasopressin injected after a; b, 10 sec, c, 30 sec, d, 5 min, e, 15 min, and f, 90 min, after vasopressin; between f and g, oxytocin (O) was injected followed immediately by vasopressin (V); g, 10 sec, h, 30 sec, i, 5 min, j, 15 min, and k, 90 min, after oxytocin and vasopressin; between k and l, third injection of vasopressin; l, 10 sec, m, 30 sec, n, 5 min, and o, 15 min, after vasopressin.

initial dose of 1 i.u./kg of vasopressin, there was a prompt rise in blood pressure followed by a temporary sharp fall (lasting for 1 to 1.5 min) and a secondary rise (lasting for 7 to 10 min). Both ST-T changes and bradycardia also occurred. After 90 min, when a similar dose of vasopressin was injected immediately following oxytocin (5 i.u./kg), both the depressor response and the ST-T changes were reduced or abolished. However, the bradycardia and pressor response were unaffected or

only slightly reduced. The third injection of vasopressin produced more marked ST-T changes than the second although less than the first. In other experiments oxytocin (5 i.u./kg) alone led to a rise in blood pressure, although Woodbury & Abreu (1944) have reported only a depressor effect with oxytocin in morphinized rabbits. It is, however, evident that oxytocin can antagonize the ST changes induced by vasopressin.

In the third series of experiments, picrotoxin (0.1 mg) was injected into a lateral cerebral ventricle in 10 normal rabbits. The animals were previously anaesthetized with pentobarbitone sodium, maintained on artificial respiration, vagotomized, and 3 to 5 mg/kg of gallamine triethiodide injected in order to prevent convulsions. Examples of the effects of oxytocin upon both the ST-T changes (A) and ventricular fibrillation (B) induced by picrotoxin are shown in Fig. 3. Following picrotoxin

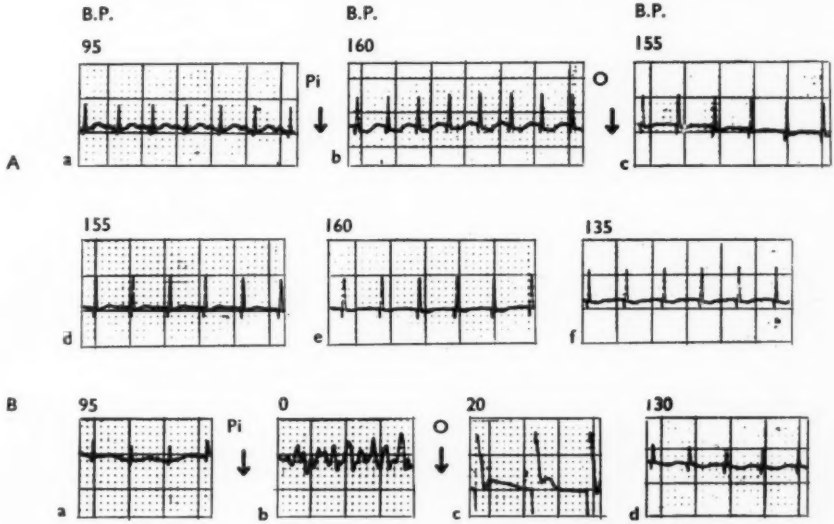

Fig. 3. Effect of oxytocin (2 i.u./kg) on the ST segment depression and ventricular fibrillation (electrocardiogram lead II) produced by injection of picrotoxin into the lateral cerebral ventricle of anaesthetized vagotomized, male rabbits paralysed with gallamine. Artificial respiration. Mean arterial blood pressure (B.P.) mm of Hg. A: 2.5 kg; a, control; b, 10 min after picrotoxin (Pi); oxytocin (O) injected after b; c, 10 sec, d, 30 sec, e, 5 min, and f, 10 min, after oxytocin. B: 3.0 kg.; a, control; b, 4 min after picrotoxin (Pi) showing ventricular fibrillation; after 3 min of fibrillation, oxytocin injected and washed with 7.0 ml. of 0.9% saline; c, 10 sec, and d, 15 min, after oxytocin.

injection, in every experiment there was a marked immediate rise in blood pressure, associated consistently with marked ST-T depression and varying degrees of cardiac arrhythmias, including sinus arrhythmia, bradycardia, tachycardia, ventricular ectopic beats and even ventricular fibrillation in different experiments. When fibrillation did not ensue early, ST-T changes with regular rhythm generally

persisted for 30 to 60 min with gradual restoration to normal. In 5 such experiments, injection of oxytocin (2.0 i.u./kg) promptly reduced the existing ST-T depression. The records of one experiment are reproduced in Fig. 3A. In these experiments ST-T depression was still absent or markedly reduced 30 min after the oxytocin.

In another group of 9 rabbits, after the onset of ventricular fibrillation, oxytocin (2.0 i.u./kg) was injected intravenously (1 to 3 min later) and rapidly washed in with 7 ml. of physiological saline. In 6 of 9 rabbits this procedure led to prompt reversal of the fibrillation with restoration of normal cardiac rhythm and blood pressure. An example of this dramatic response is shown in Fig. 3B. In the 3 unsuccessful experiments, despite injections of oxytocin fibrillation could not be reversed. It is, however, clear that oxytocin can antagonize both ST-T changes and even ventricular fibrillation under these conditions. Further studies on the anti-fibrillatory action of oxytocin under other conditions are now in progress.

DISCUSSION

The mechanism by which oxytocin exerts the above actions is still uncertain. As already pointed out, the fall in blood pressure observed following injection of pressor pituitary preparations in unanaesthetized dogs can be abolished by simultaneous injection of large quantities of oxytocin preparations. Since vasopressin is known to lead to coronary constriction, it might be assumed that the antagonistic action of oxytocin is due to a coronary vasodilator effect. Coon (1939) and Woodbury & Abreu (1944) have also reported that oxytocin exerts a coronary dilator effect in the isolated chicken heart. However, since ST-T depression under hypoxia is enhanced by coronary dilator drugs (Varma & Melville, 1961b), the antagonism of the two hormones as observed in those experiments might not involve changes in the coronary flow *per se*. Moreover, the ST-T changes induced by hypoxaemia are readily reversed by adequate oxygenation. Geiling, DeLawder & Rosenfeld (1931) and Geiling & DeLawder (1932) have observed an increase in tissue metabolism and oxygen consumption with oxytocic preparations in unanaesthetized dogs, and have also reported that vasopressin and oxytocin have opposite metabolic effects. Fraser (1937) and Brooks & Pickford (1958) have shown that oxytocic preparations can antagonize the antidiuretic effect of the pressor hormone in rats and dogs. It is therefore postulated that the protective cardiac action of oxytocin observed in these experiments might be due to a metabolic action rather than to coronary dilatation. It is conceivable that this action might enable the heart to utilize oxygen more effectively.

In connexion with the above observations the question arises whether or not this is a physiological or pharmacological action of the hormone. Relatively high doses (of 2.0 i.u./kg) are necessary to reverse ventricular fibrillation in the rabbit, but the minimal quantities required to overcome the anoxic changes under other conditions have not been determined. Assuming that the oxytocic hormone is a physiological antagonist of myocardial ischaemia, it is tempting to speculate that this might possibly be a factor in the lower incidence of coronary ischaemic attacks in women. These preliminary observations also suggest possible therapeutic usefulness of oxytocin as an antagonist of myocardial ischaemia in coronary diseases and possibly

also in preventing and reversing ventricular arrhythmias in man. It is, however, obvious that a great deal of study would be required before definite conclusions of these questions can be drawn.

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ACTION OF SOME DRUGS ON THE DORSAL ROOT POTENTIALS OF THE ISOLATED TOAD SPINAL CORD

BY

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A series of drugs which potentiate or antagonize transmission at cholinergic synapses have been tested on the slow depolarizing dorsal root potentials evoked by stimulation of adjacent dorsal and ventral roots. Some drugs acted specifically on the dorsal root potential evoked by ventral root stimulation. Dihydro-β-erythroidine, atropine and acetylcholine depressed this potential, whilst anticholinesterase substances in low concentrations potentiated and in higher concentrations depressed it. It is concluded that the results offer some evidence for a cholinergic link in the pathway responsible for the generation of depolarizing potentials in the dorsal root subsequent to ventral root stimulation.

The slow negative potential changes evoked in dorsal roots of the toad spinal cord by afferent volleys in adjacent dorsal roots were first described by Barron & Matthews (1938). Further investigations by Bonnet & Bremer (1938, 1952); by Dun & Feng (1944); by Eccles & Malcolm (1946); by Lloyd & McIntyre (1949) and by Göpfert (1956) have confirmed and extended these initial observations. Lloyd & McIntyre (1949) were able to distinguish in the dorsal root potentials (DRP) of the frog a series of potential changes, terminating in the prolonged negative potential described by Barron & Matthews (1938). They designated these deflections DRP I to V in order of temporal sequence and, of these, it is with DRP V that the present communication is concerned.

Dorsal root potentials set up by an antidromic volley in the segmental ventral root (VR–DRP) have also been described by Barron & Matthews (1938), Eccles & Malcolm (1948) and Lloyd & McIntyre (1949). The latter authors demonstrated that this potential, although being of lesser magnitude and having a longer latency, corresponded in general outline to the DRP V set up by stimulation of an adjacent dorsal root.

METHODS

All experiments were performed on lumbar segments of the isolated spinal cord of the Queensland Toad (*Bufo marinus*). The dissection and preparation bath have been previously described by Curtis, Phillis & Watkins (1961b). The cord was hemisected sagittally and was

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fixed in a small trough of 1.5 ml. capacity through which flowed amphibian Ringer solution of the following composition: NaCl 100 mM; KCl 2.5 mM; Na_2HPO_4 2.5 mM; NaH_2PO_4 0.45 mM; $CaCl_2$ 1.9 mM; $NaHCO_3$ 12 mM; glucose 2.8 mM. This solution had been equilibrated with 5% carbon dioxide and 95% oxygen before reaching the trough and had a *p*H 7.4. Pairs of platinum electrodes on the dorsal and ventral roots were used for both stimulating and recording. The dorsal root responses were displayed on an oscilloscope after amplification by an amplifier having a differential input and a time constant of 10 sec.

RESULTS

In accord with earlier investigations (Barron & Matthews, 1938; Eccles, 1947), the DRP was depressed by drugs which excite at peripheral cholinergic synapses. In addition it was found that these compounds also depressed the VR–DRP. Fig. 1A illustrates the potentials set up in a dorsal root by stimulation of the ventral

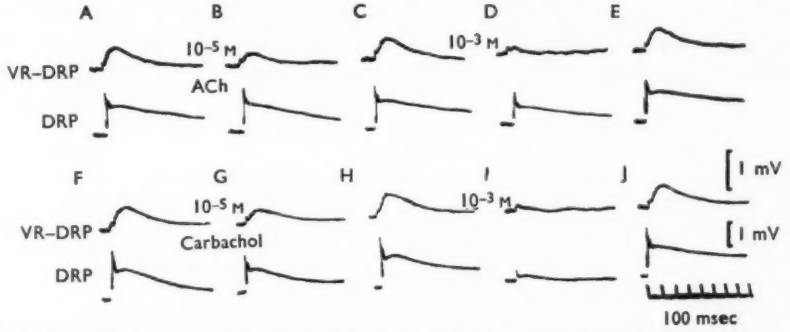

Fig. 1. VR–DRP, the potential evoked in the dorsal root (9th) by stimulation of the ventral root of the same segment. DRP, the potential evoked in dorsal root of 9th spinal segment by stimulation of dorsal root of 8th spinal segment. A, Control responses; B, after 4 min exposure to 10^{-5} M acetylcholine bromide; C, 2 min after washing; D, after 2 min exposure to 10^{-3} M acetylcholine; E, 12 min after washing. F, Control responses; G, responses after 2 min exposure to 10^{-5} M carbachol; H, recovery 8 min after washing; I, 2 min after exposure to 10^{-3} M carbachol; J, 22 min after washing. Time, 100 msec. Voltage calibration, 1 mV for VR–DRP above J, 1 mV for DRP above J.

root of the same segment (VR–DRP) and the adjacent dorsal root (DRP). A solution containing 10^{-5} M acetylcholine bromide was then passed through the bath for 4 min and the responses in Fig. 1B were recorded. The VR–DRP had been considerably reduced whereas there was very little alteration in the DRP. After washing for 4 min, the responses recovered (Fig. 1C). Two min after exposing the cord to a solution containing 10^{-3} M acetylcholine, the VR–DRP had been almost abolished and the height of the DRP had been reduced by nearly 50% (Fig. 1D). After washing, the potential took 10 min to recover (Fig. 1E).

Figs. 1F to J illustrate the effects of similar concentrations of carbachol on the same preparation. The onset of action of carbachol was slightly faster and recovery after washing slower than in the experiments with acetylcholine, but the magnitude of the depressant action of both drugs on the VR–DRP was about the same. Carbachol, however, depressed the DRP to a much greater extent than did acetylcholine. The excitant action of various amino acids (Curtis *et al.*, 1961b) on the

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toad spinal cord has been found to be associated with a decrease in the magnitude of the dorsal root potential and the generation of a negative potential change in the dorsal root (Phillis, unpublished observations). As carbachol in low concentrations produced a similar increase in the excitability of the preparation, it was not unexpected that both types of dorsal root potential were depressed. When applied in low concentrations, acetylcholine was relatively ineffective in causing an increased excitability of this preparation, so that its depressant action on the VR–DRP might be a specific effect. When applied in concentrations of 10^{-3} M or more, acetylcholine depressed both the VR–DRP and DRP and increased the ventral root reflex discharge.

Koketsu (1956) has reported that the dorsal root potential set up by ventral root stimulation was increased by eserine. This was confirmed in the present investigation. Eserine and prostigmine in low concentrations caused a small increase in magnitude and prolongation in duration of the VR–DRP, but higher concentrations depressed the response. Figs. 2A and B illustrate the DRP, VR–DRP and ventral

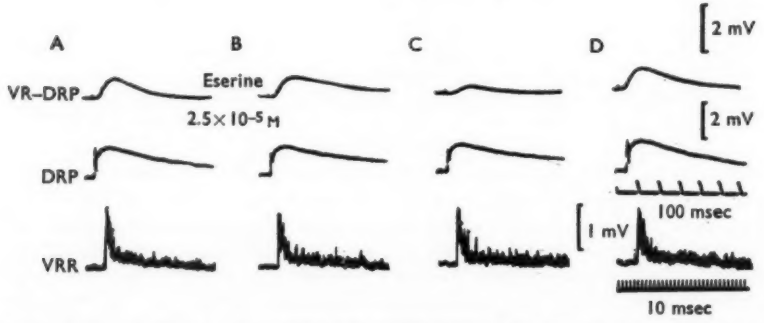

Fig. 2. Responses recorded from dorsal and ventral roots of 9th spinal segment of cord. VR–DRP and DRP are the potentials recorded from dorsal root following stimulation of corresponding ventral root and adjacent (8th) dorsal root respectively. VRR is response recorded from ventral root of segment after stimulation of 8th dorsal root. A, Control response ; B, response after exposure for 3 min to 2.5×10^{-5} M eserine salicylate ; C, response after a further 12 min of exposure to eserine solution ; D, responses after 70 min washing. Time, 100 msec for VR–DRP and DRP; 10 msec for VRR. Voltage calibration, VR–DRP, 2 mV; DRP, 2 mV; VRR, 1 mV.

root reflex response (VRR) before and after exposure of the cord to 2.5×10^{-5} M eserine salicylate. After 3 min of exposure (Fig. 2B), the VR–DRP was prolonged in duration whereas the DRP and VRR had not been altered. After 15 min of exposure, the VR–DRP had been reduced in magnitude, the DRP slightly reduced and the VRR increased slightly (Fig. 2C). After washing for 70 min, the responses recovered (Fig. 2D).

Three drugs, known to block cholinergic transmission, dihydro-β-erythroidine, hexamethonium and atropine, were tested on the magnitude of the DRP and VR–DRP. Dihydro-β-erythroidine in very low concentrations reduced the magnitude of the VR–DRP: 10 min after application of 10^{-6} M dihydro-β-erythroidine, the

control VR–DRP had been considerably reduced (Fig. 3B). After 10^{-4} M dihydro-β-erythroidine had been in the bath for a further 10 min the VR–DRP was almost abolished (Fig. 3C), but the DRP and VRR still remained virtually unaltered. Prolonged washing for 3 hr was necessary to restore the potential (Fig. 3D).

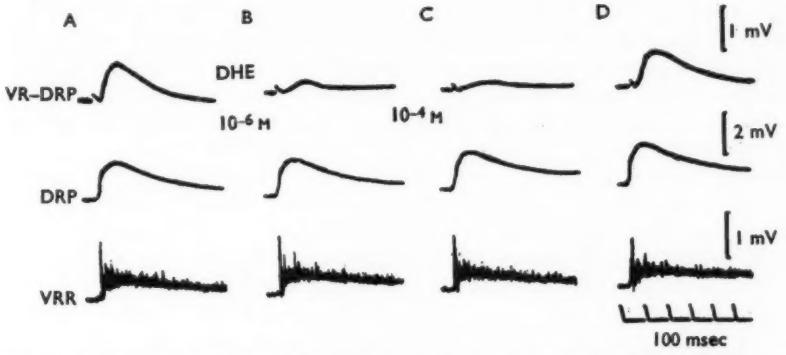

Fig. 3. Responses recorded from 9th spinal segment. See Fig. 2 for description of abbreviations. A, Control responses; B, responses after 10 min exposure to 10^{-6} M dihydro-β-erythroidine hydrobromide (DHE) ; C, after a further 10 min exposure to 10^{-4} M dihydro-β-erythroidine hydrobromide ; D, responses 3 hr after washing when VR–DRP had recovered. Time, 100 msec. Voltage calibrations, VR–DRP, 1 mV ; DRP, 2 mV ; VRR, 1 mV.

Atropine was less effective as a depressant of the VR–DRP. The minimal concentration which abolished the VR–DRP completely was 2.5×10^{-4} M (Fig. 4C), being equiactive with 10^{-5} M dihydro-β-erythroidine. Only after washing for 2 hr did the VR–DRP recover (Fig. 4D).

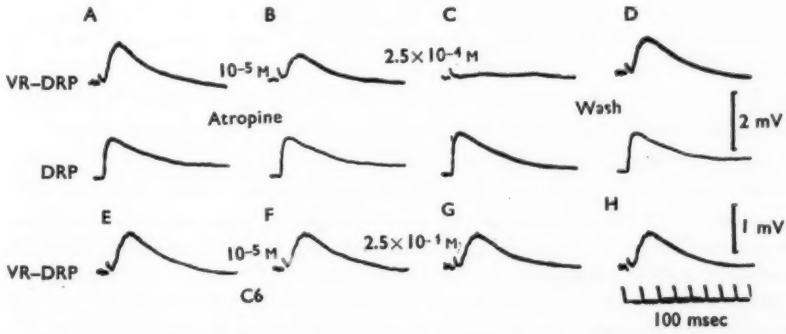

Fig. 4. Responses recorded from dorsal root of the 9th segment of spinal cord. See Fig. 2 for abbreviations. A, Control responses ; B, after 12 min exposure to 10^{-5} M atropine sulphate; C, after a further 12 min exposure to 2.5×10^{-4} M atropine sulphate ; D, 2 hr after washing. E, Control response ; F and G, after exposure for 12 min to 10^{-5} M and 2.5×10^{-4} M hexamethonium chloride (C6) respectively ; H, 1 hr after washing. Time, 100 msec. Voltage calibration, VR–DRP, 1 mV ; DRP, 2 mV.

Hexamethonium in high concentrations increased the excitability of the preparation and decreased the magnitude of both the DRP and the VR–DRP. However, the presence of 10^{-5} M and 2.5×10^{-4} M hexamethonium in the bath for periods of duration similar to those used with solutions of atropine had only a very slight depressant action on the VR–DRP (Figs. 4F and G).

The excitatory action of tubocurarine on the toad spinal cord has already been described by Eccles (1946). In most preparations in the present investigation, tubocurarine caused a reduction in the VR–DRP and the DRP, and greatly prolonged the ventral root after-discharge. After 10^{-5} M tubocurarine had been in the bath for 12 min, the responses in Fig. 5B were recorded showing that the drug

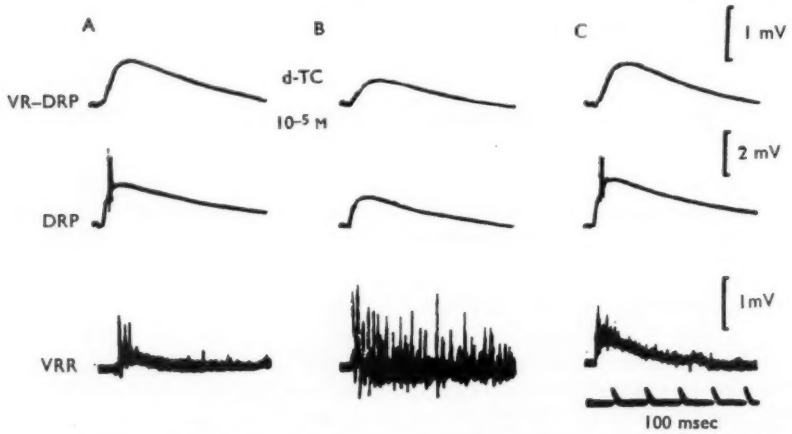

Fig. 5. Responses recorded from dorsal and ventral roots of 9th segment of spinal cord. See Fig. 2 for description of abbreviations. A, B and C, Responses before, after 12 min of exposure to 10^{-5} M tubocurarine chloride, and 1 hr after washing when the VR–DRP had fully recovered respectively. Time 100 msec. Voltage calibration, VR–DRP, 1 mV; DRP, 2 mV; VRR, 1 mV.

had a non-selective effect reducing both the VR–DRP and the DRP. The preparation was then washed and the responses recovered fully 1 hr later (Fig. 5C).

Usually the DRP was less affected than the VR–DRP, and in two preparations the VR–DRP was reduced in magnitude without a simultaneous reduction in the DRP and with very little change in the magnitude of the VRR to dorsal root stimulation. Perfusion with higher concentrations of tubocurarine in both these preparations was associated with a decrease of both the VR–DRP and the DRP and an increase in the VRR. It can be concluded, therefore, that in these cases there was evidence for a selective action on the system generating the VR–DRP.

DISCUSSION

Intracellular recording from dorsal root fibres of amphibia and mammalia (Koketsu, 1956; Eccles & Krnjevic, 1959) has shown that the dorsal root potential appears as a slow positive potential generated by a depolarization across the surface

membrane of the impaled nerve fibre. In discussing the genesis of these potentials it must be pointed out that each DRP in the present series was invariably that recorded from a root adjacent to the stimulated root and may therefore have arisen in a different fashion to the potentials generated at the pre-synaptic terminals of a stimulated root.

Available evidence indicates that dorsal root potentials are generated in the dorsal half of the spinal cord, for Pitts, McCulloch, Wall & Lettvin (1954) could only produce slow potentials in the dorsal root of cats by stimulation with a micro-electrode when the electrode was in the dorsal part of the cord, and Wall (1958) has concluded that only fibres terminating on interneurones which are concentrated in dorsal horns and intermediate nuclei appeared to be capable of generating the DRP. The ability of stimulated afferent fibres to induce potential changes in unstimulated fibres has led to the proposal that potassium ions (Barron & Matthews, 1938) or specific chemical transmitters (Fatt, 1954) released by active presynaptic fibre terminals depolarize adjacent terminals. Bonnet & Bremer (1938, 1952), Dun & Feng (1944), Eccles & Malcolm (1946), Lloyd & McIntyre (1949), Jung (1953) and Eccles & Krnjevic (1959) have suggested that interneuronal activity can contribute either by the flow of electric currents around the soma of such interneurones or by the release of transmitter from interneuronal axon terminals adjacent to primary afferent terminals. The very long duration of the DRP in amphibia suggests that sustained interneuronal activity is responsible for the later stages at least of the potential.

Koketsu (1956) concluded that the slow potential change in dorsal roots evoked by antidromic ventral root stimulation was due to the activity of motoneurones or interneurones. Its long latency (10 to 15 msec), however, apparently precludes its generation by the antidromic depolarization of the somas and dendrites of those motoneurones whose axons were stimulated. A possible route whereby interneurones in the dorsal horn of the preparation could be excited by ventral root volleys might commence along motor axon collaterals. If this be the case, then transmission at the first synapse on the pathway would be expected to be cholinergic, because other terminals of the same axons release acetylcholine at the neuromuscular junction (Dale, Feldberg & Vogt, 1936) and thus would be similar to the corresponding synapse in the cat (Eccles, Fatt & Koketsu, 1954 ; Eccles, Eccles & Fatt, 1956 ; Curtis & Eccles, 1958a and b). It was with this possible anatomical similarity in mind that this study of the pharmacology of the VR–DRP was undertaken.

The results with those drugs which depressed the VR–DRP only, and to which, therefore, may be attributed a specific action on the pathways responsible for generation of the VR–DRP, may be compared with pharmacological responses of the Renshaw cells in the cat. In low concentrations, acetylcholine, which excites Renshaw cells, specifically depressed the VR–DRP. This depressant action of acetylcholine may have been due either to a desensitization of the acetylcholine receptors (Thesleff, 1960) or to an occlusion of the response by the continuous background of excitation (Eccles, 1947 ; Curtis, Phillis & Watkins, 1961b). However, carbachol and higher concentrations of acetylcholine depressed the DRP as well as the VR–DRP and increased the general excitability of the preparation, as

evidenced by the development of depolarizing potential changes in both dorsal and ventral roots. In very low concentrations dihydro-β-erythroidine depressed the VR–DRP and was considerably more active than atropine and hexamethonium. It is also the most active of the drugs blocking cholinergic transmission on to Renshaw cells (Curtis & Eccles, 1958b ; Curtis, Phillis & Watkins, 1961a). Anticholinesterases, which prolong excitation of Renshaw cells by synaptically or iontophoretically released acetylcholine (Eccles *et al.*, 1954 ; Curtis & Eccles, 1958b), potentiated the VR–DRP when applied in very low concentrations and depressed it in higher concentrations.

While Cajal (1909) has provided histological evidence of the existence of motor axon collaterals in the amphibian spinal cord, as yet there is no physiological evidence of the existence of Renshaw-type cells (Washizu, 1960). The pharmacological findings in the present study, however, suggest that such a Renshaw-type system could be responsible for the generation of the dorsal root potential evoked by antidromic stimulation of the ventral root in amphibia.

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INTERACTIONS OF SYMPATHOMIMETIC DRUGS AND THEIR ANTAGONISTS ON THE ISOLATED ATRIUM

BY

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In the isolated guinea-pig atrium, phenoxybenzamine and other antagonists of sympathomimetic drugs and the adrenergic nerve blocking agent guanethidine inhibited the action of butyrylcholine and tyramine and potentiated the action of noradrenaline. Also in the isolated guinea-pig atrium, phenoxybenzamine and cocaine abolished the parasympathetic, and potentiated the sympathetic, effects of vagus stimulation.

In the isolated atrium phenoxybenzamine appears to mimic cocaine. Huković (1959) found that both phenoxybenzamine and cocaine potentiate the effect of sympathetic nervous stimulation in rabbit atria. Since Holtz (1959) showed that cocaine inhibits the nicotinic action of butyrylcholine, it appeared to be of interest to investigate the effect of phenoxybenzamine in this respect. It has now been found that phenoxybenzamine and other antagonists of sympathomimetic agents inhibit the action of butyrylcholine and tyramine like cocaine and potentiate the action of noradrenaline. On the other hand, McEwen (1956) found that cocaine potentiates the sympathetic effects of vagus stimulation, and a similar action of phenoxybenzamine has now been found.

The adrenergic nerve blocking drug bretylium inhibits the nicotinic action of acetylcholine but potentiates the action of tyramine (Huković, 1960). However, it has now been found that a similar agent, guanethidine, inhibits the effects of both butyrylcholine and tyramine while potentiating the effect of noradrenaline.

METHODS

The isolated guinea-pig atrium preparation has been described before (Greeff, Benfey & Bokelmann, 1959). For the vagus stimulation the right vagus nerve was fixed on a Perspex rod electrode in the bath.

The following drugs, kindly donated by the manufacturers, were used: phenoxybenzamine (Dibenzyline hydrochloride), dibenamine hydrochloride and chlorpromazine sulphoxide (S.K.F. 4260-A) from Smith, Kline & French; phentolamine (Regitine methanesulphonate) and guanethidine (Ismelin) from Ciba; piperoxane (Benzodioxane hydrochloride) from Poulenc; chlorpromazine (Megaphen) from Bayer; yohimbine from Kali Chemie; dihydroergotamine from Sandoz; opilon (acetoxythymoxyethyldimethylamine) and butyrylcholine bromide from Diwag; and noradrenaline (Arterenol) from Farbwerke Hoechst.

RESULTS

Seven minutes after a dose of phentolamine (17 μg/ml.) the action of butyrylcholine (3 μg/ml.) was prevented, the action of tyramine (3 μg/ml.) was inhibited,

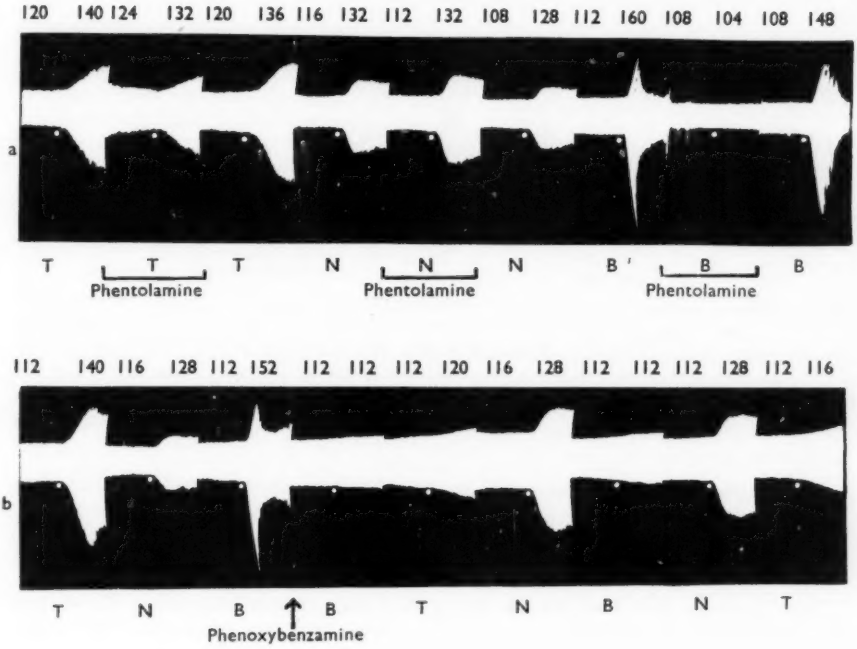

Fig. 1. Isolated guinea-pig atrium. Kymograph record taken from 3 min before until 3 min after the administration of each drug. Not recorded again until 4 min after washing out the bath. The numerals at the top of the record show the atrial rate. T=tyramine (3 μg/ml. in *a* and 7 μg/ml. in *b*); B=butyrylcholine (3 μg/ml.); N=noradrenaline (0.05 μg/ml.). During the periods marked by bars phentolamine (17 μg/ml.) was added (immediately after washing out the previous dose). In *b*, at the arrow phenoxybenzamine 25 μg/ml. was added. The bath was washed out 15 min later and the kymograph was started after an additional 4 min.

and the action of noradrenaline (0.05 μg/ml.) was potentiated (Fig. 1 A). Washing restored the activity of the drugs. The same preparation was then exposed to phenoxybenzamine (25 μg/ml.) for 15 min and the bath washed out (Fig. 1 B). The effects now were irreversible, and there was a prolonged depression of the actions of butyrylcholine and tyramine and a potentiation of that of noradrenaline.

Other antagonists of sympathomimetic drugs inhibited the response to tyramine while potentiating or not affecting that to noradrenaline. Dibenamine (25 μg/ml.) had a prolonged effect, whereas the effects of the other agents were more or less readily reversible by washing out the bath. These were: piperoxane (5 μg/ml.), chlorpromazine (3 μg/ml.), chlorpromazine sulphoxide (3 μg/ml.), dihydroergotamine (8 μg/ml.), yohimbine (5 μg/ml.), and opilon (17 μg/ml.).

Depression of the action of butyrylcholine was mostly obtained with smaller doses than were needed to inhibit the action of tyramine, for example, phentolamine

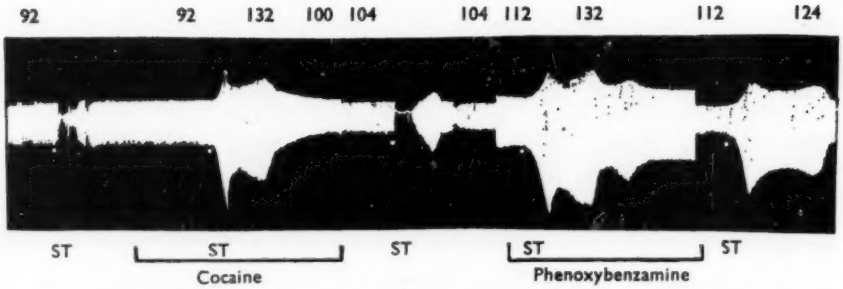

Fig. 2. Isolated guinea-pig atrium. The numerals at the top of the record show the atrial rate. At ST, the vagus was stimulated for 1.5 min with pulses of 4 mA, 1 msec duration and a frequency of 10/sec. During the period marked by the first bar cocaine 5 μg/ml. was present in the bath, and during the second bar phenoxybenzamine 25 μg/ml. was present.

(4 μg/ml.), piperoxane (3 μg/ml.), chlorpromazine (1 μg/ml.), and dihydroergotamine (4 μg/ml.).

An exposure of 5 to 10 min to 2 μg/ml. of guanethidine reversed the stimulant action of butyrylcholine, resulting in a transient depression of atrial contractions. To inhibit the action of tyramine the dose of guanethidine was increased threefold; the response to noradrenaline was potentiated under these conditions.

Fig. 2 shows that both cocaine (5 μg/ml.) and phenoxybenzamine (25 μg/ml.) converted the inhibitory effect of vagus stimulation to an excitatory one. The action of cocaine was readily reversible by washing out the bath; however, that of phenoxybenzamine persisted for a prolonged period of time.

DISCUSSION

It is puzzling that cocaine and phenoxybenzamine potentiate the effect of sympathetic stimulation (Huković, 1959) but inhibit the action of butyrylcholine. Therefore, although prevented by hexamethonium (Holtz & Westermann, 1955), the action of butyrylcholine cannot be simply a stimulation of sympathetic ganglia.

The indirectly acting sympathomimetic agents butyrylcholine and tyramine are presumably antagonized by prevention of the release of endogenous catecholamine. However, it has been found that some antagonists of sympathomimetic drugs, for example, phenoxybenzamine (Benfey, 1961), and dibenamine, phentolamine and chlorpromazine (Benfey & Varma, 1961), stimulate the heart of the spinal dog and cat, an effect prevented by reserpine or guanethidine pretreatment and apparently due to a release of endogenous catecholamine.

The sympathomimetic drug antagonists and cocaine did not convert the excitatory effect of butyrylcholine to an inhibitory one, as did reserpine (Greeff *et al.*, 1959), bretylium (Huković, 1960) and guanethidine. Furchgott (1954) found that dibenamine prevented the inhibitory action of acetylcholine in the isolated rabbit auricle. He concluded that dibenamine is an antagonist of parasympathomimetic drugs. This may also account for the prevention by phenoxybenzamine of the initial depressant effect of vagal stimulation.

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ANTIGENIC PROPERTIES OF PURIFIED FRACTIONS FROM ASCARIS LUMBRICOIDES VAR. SUUM ON NATURALLY SENSITIZED GUINEA-PIG

BY

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Purified extract of *Ascaris* injected intravenously into guinea-pigs produced a reaction very similar to the anaphylactic reaction. As shown by the ratio of deaths to survivals the toxic fraction contained in *Ascaris* extract was purified 3 to 4 times. After intravenous injection animals presented both anaphylaxis-like shock and mast cell damage similar to that observed in anaphylaxis. Perfusion of the lungs with *Ascaris* fractions released histamine. Isolated guinea-pig ileum when in contact with *Ascaris* fractions showed a response followed by desensitization. Heating the ileum to 45° C or previous desensitization to anaphylatoxin excluded the participation of the latter in this contraction. *In vitro*, sensitization from one guinea-pig could be transferred to another, using spleen extract or a 50% ammonium sulphate precipitate obtained from the spleen extract. On the basis of these results it is concluded that guinea-pigs are naturally sensitized to antigen or antigens present in *Ascaris lumbricoides*.

Shinamura & Fuji (1917) isolated from *Ascaris lumbricoides* an "albumose-peptone" which they designated as "askaron," a very toxic substance for dogs. In 1939 Machebouef & Mandoul showed that deproteinized extract from *Ascaris megalocephala*, when injected intravenously into guinea-pigs, produced a reaction reminiscent of anaphylaxis. Bier (1939) showed that *Ascaris lumbricoides* contained a substance very active in producing an anaphylaxis-like shock in guinea-pig. Rocha e Silva & Grãna (1946), using a deproteinized and dialysed extract from *Ascaris lumbricoides*, were able to produce fatal shock in dogs and guinea-pigs. Further purification of this material led them to identify the active fraction as a proteose of high molecular weight. Nakajima (1954), after heating to 80° C an aqueous extract of *Ascaris lumbricoides*, purified it by alcohol precipitation, and obtained an active fraction containing nitrogen and phosphorus. Högberg, Thufvesson & Uvnäs (1956), precipitating *Ascaris* extract in alcohol followed by passage through an Amberlite IRC-50 XE-64 column, obtained an active fraction which released histamine from perfused paw of the cat. Recently, Gazzinelli, Guia, Neves, Pudles, Beraldo & Dias da Silva (1961) showed that the active fraction of the *Ascaris lumbricoides* was a complex substance containing carbohydrate and protein.

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Furthermore, they suggested that the guinea-pig is naturally sensitized to *Ascaris lumbricoides*.

In the present paper the chemical analysis of the active fraction was extended and its further purification explored. In addition the mechanism of action of this fraction on the guinea-pig was studied.

METHODS

Guinea-pigs of either sex, body weight 200 to 400 g, were used without any previous sensitization.

Intravenous injection into unanaesthetized guinea-pig. The injections were made using the veins of the front legs by means of a 1 ml. tuberculin syringe. The volume of the solutions injected did not exceed 1 ml.

Mast cell observation. The lungs were fixed as described by Mota & Vugman (1956). The mast cell number was assessed by counting thirty fields, at a magnification of 900, and the reduction in the number of the cells was expressed as % of the control.

Perfusion of the lungs. The animals were anaesthetized with ether. The trachea was cannulated and the thorax opened. A polythene cannula was inserted into the pulmonary artery and the perfusion started. Another cannula was tied into the left ventricle for the collection of perfusates. The preparation (lungs, thorax and head) was kept at about 38° C. The lungs were perfused with Tyrode solution at 38° C, at a rate ranging between 1 and 1.5 ml./min. After washing the lungs free of blood as completely as possible two 5 ml. samples were collected as controls. The perfusion was stopped and 0.5 to 1 ml. of a solution, containing *Ascaris* fraction, was injected through the cannula inserted into the pulmonary artery, by means of a tuberculin syringe. The injected solution was allowed to remain in contact with the pulmonary tissues. Five min later the perfusion was continued until only traces of histamine could be detected in the perfusates.

Histamine assays were performed on the atropinized guinea-pig ileum according to Feldberg & Talesnik (1953). All histamine values are given as base.

Purification of active fractions from Ascaris lumbricoides. *Ascaris lumbricoides* collected from hogs were treated according to the method described by Rocha e Silva & Grãna (1946). 875 g of *Ascaris* yielded 850 mg of an active fraction, called SB_1; 765 mg of this material was dissolved in 60 ml. 1 M acetate buffer of *p*H 5.2 at 4° C; ammonium sulphate was added until a flocculent precipitate began to form. The precipitate was centrifuged at 3,000 r.p.m. at 4° C, the supernatant collected and dialysed against distilled water for 24 hr in the cold room and then lyophilized. 405 mg of the lyophilized material was dissolved in 6 ml. 0.01 M phosphate buffer of *p*H 7.5 and was run through a column (1 × 55 cm) of hydroxyapatite. When the same buffer was used for elution and 3 ml. eluates were collected, the active substance appeared in the eluates nos. 18 to 30. These eluates also showed the highest absorption for protein at 280 mμ. Further elution with higher ionic strengths gave other protein peaks which still contained some activity. The yield of active eluates nos. 18 to 30, after 48 hr dialysis against distilled water and lyophilization, was of 69 mg (fraction SB_3). The rechromatography of the fraction SB_3, on a column of Amberlite IRC-50, XE-64, showed only one protein peak. Total nitrogen was estimated by micro-Kjeldahl and Nesslerization. For protein determination the method of Lowry (1951) was employed using serum as standard. The ultraviolet absorption method of Warburg and Christian (1941) was used for comparison. Organic and inorganic phosphorus were determined according to Nakamura (1952), the reducing sugars by the method of Somogyi (1945). Each fraction was hydrolysed in a sealed tube with 3 N hydrochloric acid for 5 hr in a boiling water bath. After neutralization, an aliquot was taken and estimated. The results were expressed in terms of glucose. The amino sugars were estimated by the method of Elson and Morgan (1933) and the results expressed in terms of glucosamine. The analytical data presented in Table 1 show the maximum and minimum amounts of proteins, reducing sugars and nitrogen obtained from seven different *Ascaris* batches.

TABLE 1

CHEMICAL ANALYSIS OF FRACTIONS SB_1 AND SB_3 FROM *ASCARIS LUMBRICOIDES*

Fraction	Protein (%) Lowry	Protein (%) 280/260 mμ/mμ	Reducing sugars (%)	Amino sugars (%)	Nitrogen (%)	Total phosphorus (%)
SB_1	27·4–29	33	15–23·3	6·6	7·7–8·7	1·18
SB_3	7·6–11	7·7–10	35–42	14	3·4–5·5	1·80

The analytical data of amino sugars and total phosphorus refer only to the preparations containing the minimum amount of nitrogen, protein and reducing sugars. In all purified fractions a positive Sakaguchi, a feeble Molisch and negative Ehrlich reaction for sialic acid were found (Blix, 1936). The test for free sulphydryl groups was also negative.

Substances used. Histamine diphosphate (Nutritional Biochemicals Corporation). Diphenhydramine hydrochloride (Benadryl, Parke, Davis). Anaphylatoxin was prepared according to Rocha e Silva & Aronson (1952).

RESULTS

Intravenous injection of Ascaris *fractions.* The majority of the animals, when injected with purified fractions of *Ascaris*, showed a reaction very similar to anaphylactic shock. The activity of the fractions SB_1 and SB_3 was determined by recording the ratio of deaths to survivals, in groups of 6 animals, as shown in Table 2.

TABLE 2

RELATION OF DOSE (μG/100 G) TO MORTALITY FOR GUINEA-PIGS INJECTED WITH SB_1 AND SB_3 FRACTIONS

Ascaris fraction	Total weight (μg)	Protein (μg)	Residue (μg)	Mortality ratio
SB_1	50	15	35	0/6
	133	40	93	3/6
	200	60	140	5/6
SB_3	132	10	122	4/6
	264	20	244	5/6

TABLE 3

REDUCTION IN MAST CELL NUMBER IN GUINEA-PIG LUNG AFTER INTRAVENOUS INJECTION OF 30 μG/100 G *ASCARIS* FRACTION SB_3

Mast cell numbers are the means of thirty microscopical fields at a magnification of ×900

Guinea-pig	Injected with	Number of mast cells per field	(means)	% of reduction
1	1 ml. sodium chloride 0·9%	14		
2		10		
3		12	14	—
4		18		
5		16		
6	SB_3 in 1 ml. sodium chloride 0·9%	2		
7		1		
8		5	3	78·5
9		3		
10		4		

Mast cell damage. Microscopic examination of the lungs of the guinea-pigs receiving intravenous injection of *Ascaris* fractions which died in shock showed constant and well-defined alterations in the mast cells. These were characterized by disappearance of granules (degranulation) and a consequent reduction in the number of stainable mast cells. As may be seen in Table 3, the *Ascaris* fractions produced a 78.5% reduction of the number of the mast cells.

Histamine release from the perfused guinea-pig lungs. In 6 experiments, perfusion of the isolated guinea-pig lungs with purified fractions from *Ascaris lumbricoides* was accompanied by the release of histamine 0.9 to 5.8 μg. Fig. 1 shows the output of histamine following the injection of SB_3 fraction. Ten min after the injection, 1.5 μg (46%) of the liberated histamine appeared in the perfusate and 40 min after the injection only traces of histamine were found in the perfusate. As a rule, the second injection of same amount or even of greater amounts of the *Ascaris* fraction yielded only about one-half of the total histamine released by the first injection.

Experiments with the isolated guinea-pig ileum. Guinea-pig ileum fragments 2.5 to 3 cm long were suspended in 10 ml. atropinized Tyrode solution. The addition of 1 to 10 μg of the *Ascaris* fractions to the bath containing the guinea-pig ileum

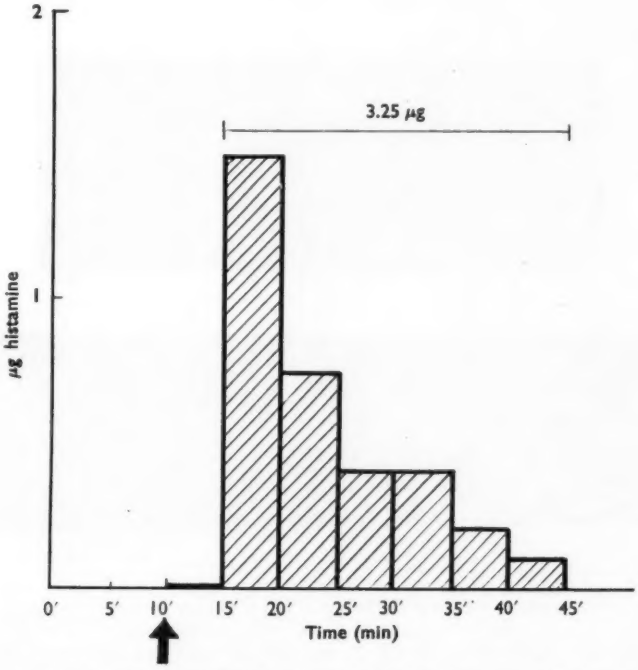

Fig. 1. Histamine release from perfused guinea-pig lungs. The arrow indicates the injection of SB_3 fraction 28 μg into the cannula.

evoked, after a period of latency about 60 to 90 sec, a strong contraction. A second addition of the same amount was either ineffective or produced a small effect, followed by a refractory state (desensitization) to a further addition of the *Ascaris* fractions. The response of the ileum and its desensitization was observed in 30 out of 35 different preparations tested.

In order to verify whether the desensitization of the ileum would occur also *in vivo*, 7 guinea-pigs were injected intravenously with sublethal doses of *Ascaris* fractions. Two days later the ilea were suspended in an organ bath and challenged as described above.

None of the ilea of the desensitized guinea-pigs responded when *Ascaris* fraction was added to the perfusion bath.

Possible participation of anaphylatoxin. Mongar & Schild (1957) showed that previous heating at 45° C of guinea-pig tissues inhibited the anaphylactic reaction *in vitro*. When anaphylatoxin was used, however, this effect was not observed (Mota, 1959).

In order to investigate whether the contraction of the guinea-pig ileum produced by *Ascaris* fractions was due to an anaphylatoxin-like substance present in the *Ascaris*, it was decided to try the effect of heat on this reaction. To test this hypothesis two fragments of the same ileum were taken. The first one (control) was

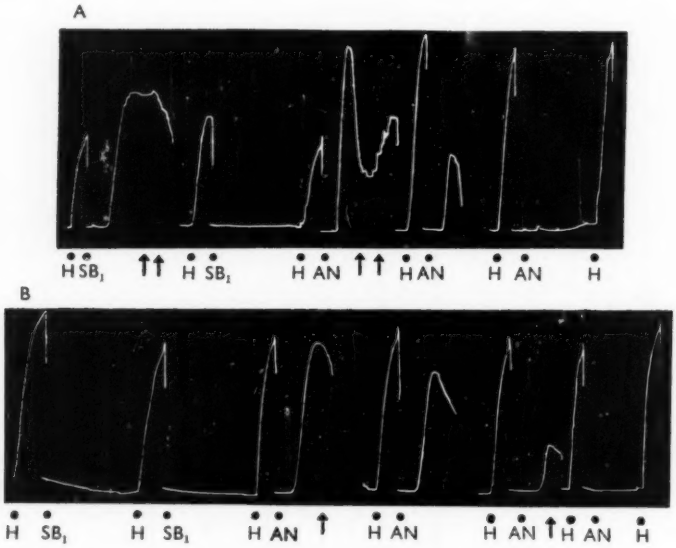

Fig. 2. Guinea-pig ileum preparation suspended in 10 ml. Tyrode solution. *Tracing A*, H=0.1 μg histamine; SB_1=100 μg *Ascaris* fraction; AN=0.1 ml. anaphylatoxin. *Tracing B*, The ileum was previously heated at 45° C for 15 min; H=0.1 μg histamine; SB_1=100 μg *Ascaris* fraction. AN=0.1 ml. anaphylatoxin. The arrows indicate washings with Tyrode solution. The substances were added to the bath at 2 min intervals.

desensitized to *Ascaris* fraction (SB_1). Even in this condition, the preparation produced a very strong contraction when anaphylatoxin was added to the bath (Fig. 2, A). The second fragment of the ileum was heated at 45° C, for 15 min, suspended in Tyrode solution at 35° C, and the effect of *Ascaris* fraction (SB_1) and anaphylatoxin tested. The heated fragment gave no response to *Ascaris* fraction, but gave a strong response to anaphylatoxin (Fig. 2, B). Besides, previous desensitization of ileum to anaphylatoxin does not affect the response to *Ascaris* fraction. As was observed by Mota (1959), in a guinea-pig ileum desensitized to anaphylatoxin, a subsequent addition of the specific antigen still produces the anaphylactic response.

Passive sensitization in vitro. As the participation of anaphylatoxin in the reaction produced by *Ascaris* fractions was excluded, the possibility was examined that an antigen-antibody reaction was involved in this contraction. To test this, fragments of guinea-pig ileum, previously made refractory to *Ascaris* fractions, were incubated at 35° C for 2 hr with serum collected from a guinea-pig whose ileum was tested and found to be reactive to the *Ascaris* fractions. After incubation, the ileum was challenged with *Ascaris* fraction and it was observed that in 2 out of 6 experiments the sensitivity had been transferred from the guinea-pig serum to the ilea. In an attempt to increase the frequency of the passive sensitization, guinea-pig spleen extract was tested in view of experiments by several authors showing that spleen is an organ which produces and stores antibodies (Stavitsky, 1955 ; McKenna & Stevens, 1957). Spleen extracts were prepared in a glass homogenizer and distilled water was added in order to make a final concentration of 10%. After centrifugation the supernatant was collected ; sodium chloride and glucose were added in the same proportion as in the Tyrode solution. This extract was used for passive sensitization as follows: guinea-pig ilea previously desensitized to *Ascaris* fraction were incubated with the supernatant for 2 hr at 35° C. They were then washed with Tyrode solution and challenged by adding *Ascaris* fractions to the bath. In 8 out of 12 experiments the ileum contracted when in contact with *Ascaris* fraction. A second or third addition of *Ascaris* fraction caused only a small response of the ileum or no response at all. Furthermore, the precipitate obtained from spleen extract by ammonium sulphate retained the ability of transferring passive sensitization, as can be seen in the following experiment: 20 ml. spleen extract was precipitated by adding ammonium sulphate to 50% saturation. The precipitate was dissolved in 10 ml. distilled water and dialysed against tap water for 48 hr at room temperature and the dialysis was continued against saline at about 4° C for 10 to 12 hr. This material was used for the *in vitro* passive sensitization. Fig. 3 illustrates one of these experiments.

In order to verify the specificity of the passive sensitization spleen extracts were prepared from guinea-pigs with ilea insensitive to *Ascaris* fractions (negative Schultz-Dale-like reaction). In 5 experiments in which different spleen extracts were used sensitization could not be transferred to the ileum. In addition, the specificity of the *Ascaris* fractions to produce contractions of the passively sensitized ileum was also investigated using other antigens. Fig. 4 shows that no contraction of the ileum was obtained when horse serum or ovalbumin was used, although the ileum contracted when *Ascaris* fractions were added to the bath.

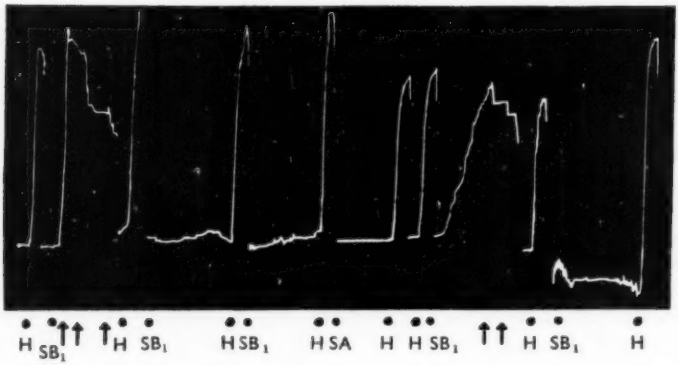

Fig. 3. Guinea-pig ileum suspended in 10 ml. Tyrode solution: H=0.05 μg histamine. SB_1=130 μg *Ascaris* fraction. SA=10 ml. spleen extract precipitate (50% ammonium sulphate saturation) dissolved in distilled water and dialysed against saline (see text), allowed to remain in the bath for 2 hr. The arrows indicate washings with Tyrode solution. The substances were added to the bath at 2 min intervals.

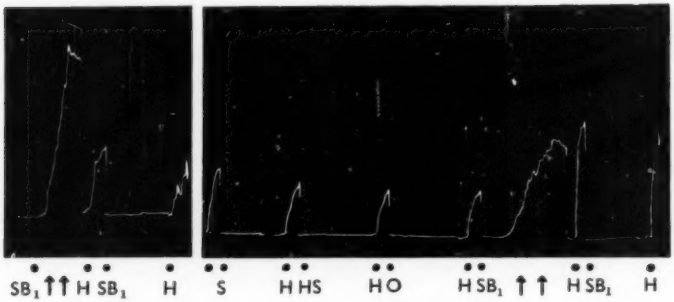

Fig. 4. Guinea-pig ileum preparation in 10 ml. Tyrode solution: SB_1=130 μg *Ascaris* fraction H=0.1 μg histamine. S=10 ml., 10% spleen extract, allowed to remain in the bath for 2 hr. HS=0.1 ml. horse serum. O=1 mg ovalbumin. The arrows indicate washings with Tyrode solution. The substances were added to the bath at 2 min intervals.

DISCUSSION

The intravenous injection of *Ascaris* extract produced in the guinea-pig a fatal anaphylaxis-like reaction. These experiments confirm those described by Bier (1939) and Rocha e Silva & Grãna (1946).

The perfusion of the guinea-pig lungs with purified *Ascaris* fractions led to a release of histamine which ranged between 0.9 and 5.8 μg. These results agree with those obtained by Bartosch, Feldberg & Nagel (1932) for guinea-pig lungs perfused with specific antigen. In addition, the alterations of mast cells produced by purified

Ascaris fractions were very similar to those induced by anaphylatoxin (Mota, 1959) or specific antigen in sensitized guinea-pig (Mota & Vugman, 1956).

The experiments with isolated ileum showed that *Ascaris* fractions are able to produce a strong contraction of this organ, indistinguishable from that induced in anaphylaxis (Schultz, 1910a, b ; Dale, 1913) or by anaphylatoxin (Rothschild & Rocha e Silva, 1954). However, the results with the ileum heated to 45° C or previously desensitized to anaphylatoxin excluded the participation of the latter, and suggested an antigen-antibody reaction in the mechanism of this contraction (Mongar & Schild, 1957 ; Mota, 1959).

This problem was further studied by sensitization of ilea by spleen extract *in vitro*. It was also observed that a 50% ammonium sulphate precipitate obtained from spleen extract retains the ability of sensitizing the ileum. The possibility of spontaneous or non-specific resensitization, in the absence of sensitive spleen extract, was excluded by experiments on spleen extracts from guinea-pigs with ilea insensitive to the *Ascaris* fractions. Our experiments therefore support the view that a passive sensitization by antibodies present in spleen extract was obtained. These results do not mean that spleen extract is richer in antibodies than serum, for in these experiments no comparative study between serum and spleen extract was performed.

Those guinea-pigs in which the intravenous injections of the *Ascaris* fractions did not cause any effect also showed no mast cell damage. In such guinea-pigs the ilea proved insensitive to the *Ascaris* fractions and the release of histamine from the lungs did not occur ; furthermore, the spleen extracts obtained from these animals were unable to transfer the passive sensitization.

These findings suggest that all effects produced by *Ascaris* extracts on the guinea-pig depend on a natural sensitization of the animals to antigen or antigens present in the *Ascaris lumbricoides*.

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EFFECT OF GUANETHIDINE IN REVEALING CHOLINERGIC SYMPATHETIC FIBRES

BY

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Guanethidine abolished the inhibitory response of segments of rabbit intestine to stimulation of the sympathetic nerves which accompany the mesenteric arteries. In the majority of experiments a motor response of the intestinal segment was then revealed; it was more readily observed in intestinal segments from young than from adult rabbits. The motor response of the intestine to sympathetic stimulation after guanethidine was blocked by atropine. It was not blocked by hexamethonium and was present in rabbits in which the vagal innervation to the small intestine had been sectioned. In the cat isolated atria, guanethidine blocked the accelerator response to sympathetic nerve stimulation and revealed a response resembling that to stimulation of the vagus. It was concluded that guanethidine blocked the release of noradrenaline and thus revealed the response to the direct action of acetylcholine released from cholinergic sympathetic nerves.

Cholinergic fibres occur so frequently in sympathetic nerves that they may be considered as typical rather than exceptional. Their existence provides a clue to the mechanism by which the impulse arriving at the sympathetic nerve ending may liberate noradrenaline (Burn & Rand, 1959b, 1960b).

The detection of cholinergic sympathetic fibres has been facilitated by the use of reserpine. When animals are injected with reserpine the noradrenaline content of the tissues is considerably reduced (Bertler, Carlsson & Rosengren, 1956; Burn & Rand, 1958a; Burn & Rand, 1959a), and consequently the response to sympathetic nerve stimulation which resembles the response to noradrenaline is reduced or abolished (Bein, 1953; Muscholl & Vogt, 1958; Burn & Rand, 1958b; Burn, Leach, Rand & Thompson, 1959; Trendelenburg & Gravenstein, 1958), and in some cases replaced by a response which can be shown to be due to acetylcholine liberation. Thus, in reserpine-treated rabbits, Gillespie & MacKenna (1959, 1961) found that stimulation of the sympathetic nerves to the isolated colon produced contraction instead of inhibition, and Huković (1959) found that stimulation of the accelerans nerve to the isolated atria produced slowing instead of an increase in rate. Burn & Rand (1960a) demonstrated cholinergic fibres in the sympathetic supply to the nictitating membrane, spleen, and uterus of the cat, and to the rabbit ear.

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The report by Boura & Green (1959) on the pharmacology of bretylium contains data which suggest that bretylium not only prevents the release of noradrenaline but also unmasks the direct (muscarinic) action of acetylcholine from cholinergic sympathetic fibres on the innervated tissue. Thus, after bretylium, stimulation of the nervi accelerans to the cat heart no longer increased but decreased the rate, and this slowing of the heart could be blocked by atropine. Similarly, Huković (1960) found that bretylium blocked the vasoconstrictor response to sympathetic nerve stimulation in the perfused rabbit ear and unmasked a dilator response. Blair, Glover, Kidd & Roddie (1960) found that bretylium blocked vasoconstriction in the human forearm caused by sympathetic adrenergic fibres but left unaffected vasodilatation caused by cholinergic sympathetic nerves excited by emotion. The effect of bretylium in blocking adrenergic but not cholinergic fibres in sympathetic nerves can explain syncope on exertion in patients who are receiving bretylium to control hypertension (Brandon, 1960).

Another drug which, like bretylium, blocks the usual response to stimulation of sympathetic "adrenergic" nerves is guanethidine (Maxwell, Plummer, Schneider, Povalski & Daniel, 1960). This paper deals with some observations on the presence, as revealed by guanethidine, of cholinergic fibres in the sympathetic nerves to the rabbit intestine and to the cat atria.

METHODS

Isolated rabbit intestine. Segments of isolated rabbit intestine with their sympathetic nerves intact were prepared by the method of Finkleman (1930). Rabbits were stunned and bled out, the abdomen opened and a segment of intestine with its mesentery was spread out. A ligature was placed around a suitable branch of mesenteric artery about 2 to 4 cm from the gut. A length of intestine 1.5 to 3 cm long which contained the branches of the selected mesenteric artery was excised and suspended in McEwen (1956) solution in a 70 ml. or a 100 ml. organ bath at 37° C. The bath was bubbled with 5% carbon dioxide in oxygen. In a few experiments Krebs bicarbonate solution or Tyrode solution was used. The artery together with its accompanying sympathetic nerves was passed through a channel containing bipolar platinum electrodes placed with the cathode nearest to the gut. The electrodes were arranged so that there was no interference with the movement of the intestinal segment in the bath. In some experiments the intestinal segments were arranged for transmural electrical stimulation (Paton, 1957). The lower end of the segment was tied over a glass tube so that the lumen enclosed a platinum wire 2 cm long, which was the internal electrode. The electrodes connected to the sympathetic nerve were joined together to form the external electrode. The periarterial sympathetic nerves were stimulated with 5 to 15 V square wave pulses from a constant voltage output electronic stimulator; other details of stimulation are given below in the section on RESULTS. The longitudinal contractions of the gut were recorded by a lever writing on smoked paper.

Vagotomy. The vagus nerves were divided by aseptic operation in rabbits anaesthetized with intravenous thiopentone sodium (20 mg/kg) and ether. Two methods of causing the degeneration of vagal fibres to the intestine were used: (a) the right vagus was divided in the neck, or (b) both vagi were divided in the abdomen at the point where they accompany the oesophagus immediately before it enters the stomach. Attempts were made to divide both vagi in the neck, but it was found that the rabbits survived for only 12 to 24 hr in either 1 or 2 stage operations. This has been reported previously (Lorber, 1939).

Cat atria. Isolated atria from young cats with their sympathetic nerves attached were prepared as described by Chang & Rand (1960); in addition the vagus nerves were attached (Burn & Rand, 1958a).

Drugs. The following drugs were used: guanethidine (2-(octahydro-1-azocinyl)-ethyl-guanidine sulphate), atropine sulphate, hyoscine hydrobromide, hexamethonium bromide, acetylcholine chloride, histamine acid phosphate, 5-hydroxytryptamine creatinine sulphate (serotonin), physostigmine salicylate, neostigmine methylsulphate, nicotine bitartrate, 2,6-xylyl ether of choline bromide (TM10), bretylium tosylate; the doses of these drugs are expressed as the salt. Reserpine, adrenaline and noradrenaline were used in doses given as the base, and doses of adenosine triphosphate are given as the acid.

RESULTS

Effect of guanethidine on stimulation of sympathetic nerves. Stimulation of the sympathetic nerves accompanying a mesenteric artery produced inhibition of the pendulum movements in the isolated segment of rabbit ileum. After the addition of guanethidine (1 μg/ml.) to the bath the inhibitory response was gradually lost and was usually replaced by a motor response. In a total of 35 segments of intestine from 27 rabbits the reversal of the response to sympathetic nerve stimulation from an inhibition to motor response has been observed in 25 segments. The exact nature of the motor response varied somewhat in various experiments (compare Figs. 1, 2, 3 and 5), but, in general, it resembled the response to a small dose of acetylcholine in that particular experiment. Atropine and hyoscine, in doses which abolished the response to acetylcholine, considerably reduced or abolished the motor response to sympathetic stimulation (Figs. 1, 2 and 3).

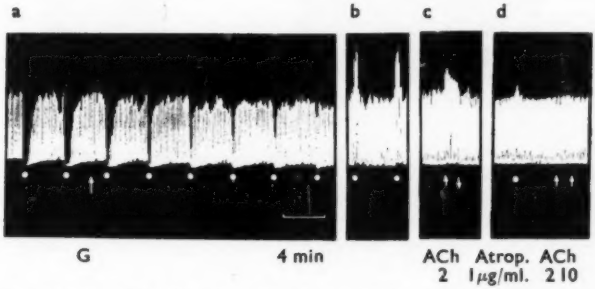

Fig. 1. Isolated rabbit ileum in 100 ml. bath. Periarterial sympathetic nerves stimulated at the white dots with 2 msec pulses for 14 sec every 4 min, in *a* at 50/sec, and in *b* and *d* at 10/sec. Guanethidine, 1 μg/ml. at G, blocked the inhibitory response. In *b*, 120 min later, stimulation produced a motor response. In *c*, 2 μg acetylcholine (ACh) produced a motor response. Atropine 1 μg/ml. in *d* blocked the motor responses to sympathetic stimulation and to acetylcholine.

In some experiments the movements of the intestine *in situ* were recorded. Stimulation of the periarterial sympathetic nerves at 20/sec and 50/sec produced inhibition. After an intravenous injection of 0.5 to 5 mg/kg guanethidine the inhibitory response was blocked and there was a pronounced motor response to sympathetic stimulation which was blocked by 10 mg/kg atropine.

Since the motor response to sympathetic stimulation after guanethidine appeared to be mediated by cholinergic fibres, attempts were made to enhance the response with anticholinesterases. The addition of eserine or neostigmine to the bath resulted

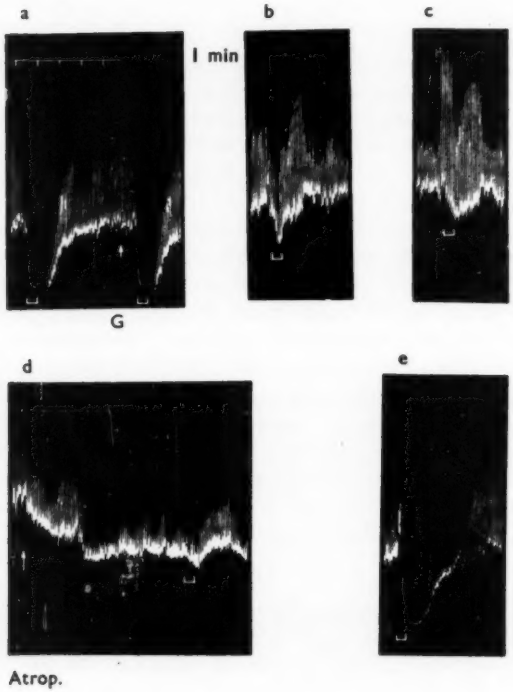

Fig. 2. Isolated rabbit ileum in Tyrode solution. The periarterial sympathetic nerves were stimulated with 0.5 msec pulses at 50/sec for 30 sec indicated by |__|. In *a*, 1 μg/ml. of guanethidine (G) was added to the bath. In *b*, 20 min later, the smaller inhibitory response was followed by a motor response. In *c*, 35 min after guanethidine, there was a pure motor response which was antagonized by atropine (Atrop., 2 μg in 130 ml. bath) in *d*. The bath was then washed repeatedly during 110 min which restored the inhibitory response in *e*.

in an increase in tone, and the intestinal segment was then insensitive to acetylcholine, noradrenaline and to sympathetic nerve stimulation. Burn (1952) illustrates an experiment in which eserine did not produce a rise in tone when glucose was absent from the bath fluid. Therefore we carried out an experiment in a glucose-free solution, but the effects of drugs and of sympathetic stimulation were then markedly reduced, and after eserine the response to acetylcholine was not increased.

The effect of hexamethonium on the motor response was somewhat variable. In the experiment shown in Fig. 3 a concentration of 1 mg/ml. did not affect the motor response, whereas in another experiment hexamethonium in a concentration of 10 μg/ml. reduced the motor response. Garry & Gillespie (1955) found that 50 μg/ml. hexamethonium always caused some block of the response of the colon to parasympathetic nerve stimulation, and 0.5 mg/ml. blocked it completely. Gillespie & MacKenna (1961) found that 0.2 mg/ml. hexamethonium blocked the

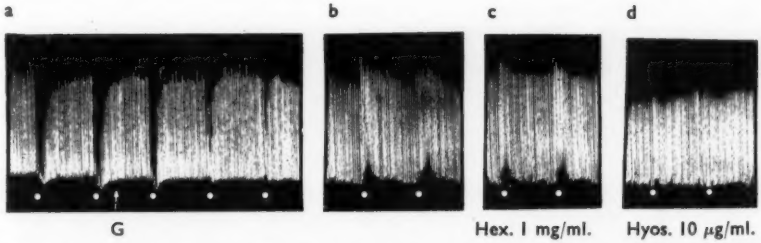

Fig. 3. Isolated rabbit ileum. The sympathetic nerve was stimulated with 2 msec pulses at 50/sec for 20 sec every 3 min at the white dots. In *a*, guanethidine 1 μg/ml. was added to the bath at G, and in *b*, 20 min later, a motor response was present. Hexamethonium 1 mg/ml. in *c* did not block this response, but 10 μg/ml. of hyoscine in *d* did.

motor response to sympathetic nerve stimulation seen in the isolated colon from reserpinized rabbits. They concluded that after reserpine sympathetic stimulation causes an activation of parasympathetic pathways. Since larger doses of hexamethonium than those used by Gillespie and his colleagues did not regularly block the motor responses to sympathetic stimulation revealed by guanethidine in the isolated ileum, it is unlikely that this motor response is due to parasympathetic nerves.

After guanethidine had caused an alteration of the response to sympathetic stimulation from inhibition to excitation it was difficult to restore the original effect by washing. One experiment in which the inhibitory response was successfully restored is shown in Fig. 2, in which the bath was washed 10 times at 5 min intervals ; it is interesting to note that in this experiment the restored inhibition was preceded by a contraction.

The abolition of the motor response by atropine and by hyoscine is not a final proof that it is due to cholinergic nerves. The following experiments were undertaken to determine whether other substances may have caused the motor response.

Concentrations of mepyramine (0.5 μg/ml.) which completely blocked the response of the intestine to histamine (1 to 10 μg/ml.) did not affect the motor response to sympathetic stimulation revealed by guanethidine. Larger amounts of mepyramine reduced the motor responses both to acetylcholine and to sympathetic stimulation.

It is unlikely that the motor response to sympathetic nerve stimulation can be due to 5-hydroxytryptamine, since 50 times the concentration of atropine was needed to block it than to block an equiactive dose of acetylcholine on the rabbit intestine, and the antagonism of 5-hydroxytryptamine by atropine was only shown when atropine remained in the bath, whereas the atropine block of the motor responses to sympathetic stimulation and to acetylcholine was difficult to reverse by washing.

Adenosine triphosphate in concentrations of 1 to 10 μg/ml. produced only an inhibition of rabbit intestine, and smaller concentrations had no action.

Effect of guanethidine on responses to acetylcholine and noradrenaline. When the concentration of guanethidine in the bath was increased to 10 μg/ml. the motor response of the ileum to sympathetic stimulation was reduced or abolished. This

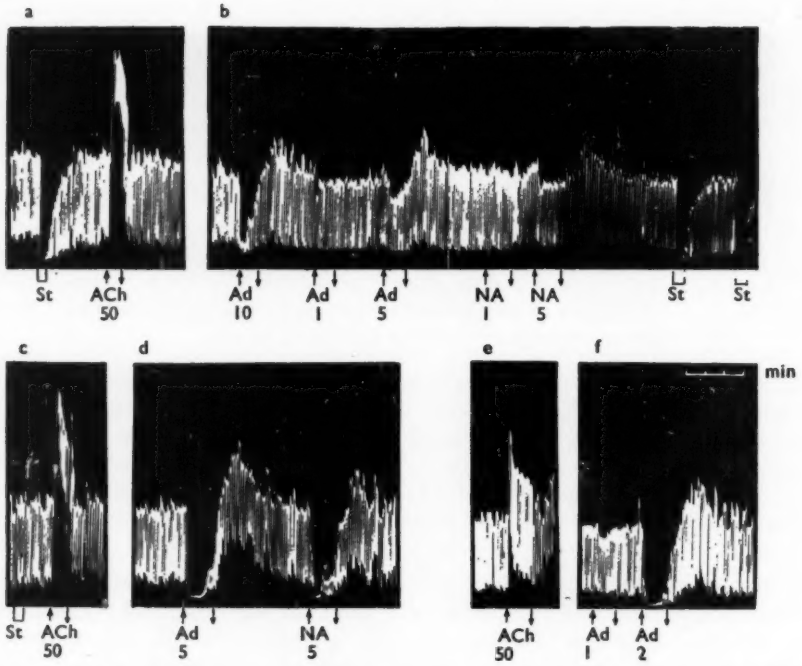

Fig. 4. Effect of guanethidine on response of ileum to adrenaline (Ad), noradrenaline (NA), acetylcholine (ACh), and sympathetic stimulation at 50/sec for 20 sec (St). Drugs were added to the bath at ↑ and washed out at ↓. The numerals refer to the doses in μg added to a 70 ml. bath. Observations were made before guanethidine in *a* and *b*, after 1 μg/ml. guanethidine in *c* and *d*, and after 10 μg/ml. guanethidine in *e* and *f*.

appears to be due to an anti-muscarinic action of guanethidine, since the motor response to acetylcholine was also diminished (Fig. 4e). The contraction of the colon in response to nicotine was diminished by guanethidine (Fig. 7). The inhibitor response to adrenaline and noradrenaline was potentiated by guanethidine. Fig. 4b shows that, before guanethidine, both adrenaline (5 μg) and noradrenaline (5 μg) produced a very slight decrease in amplitude of the pendulum movements, but after 1 μg/ml. of guanethidine, when the inhibitor response to sympathetic stimulation had been abolished, the same doses of adrenaline and noradrenaline now produced complete inhibition of movements and a decrease in tonus (Fig. 4d). After 10 μg/ml. of guanethidine the effects of adrenaline (Fig. 4f) and noradrenaline were still further potentiated.

Stimulus parameters. Garry & Gillespie (1955) have determined the stimulus parameters for optimum responses of the rabbit isolated colon to stimulation of the sympathetic and parasympathetic nerves. They found that the optimum frequency of sympathetic nerve stimulation for the inhibitory response was 100/sec and the optimum frequency for the motor response to parasympathetic nerve

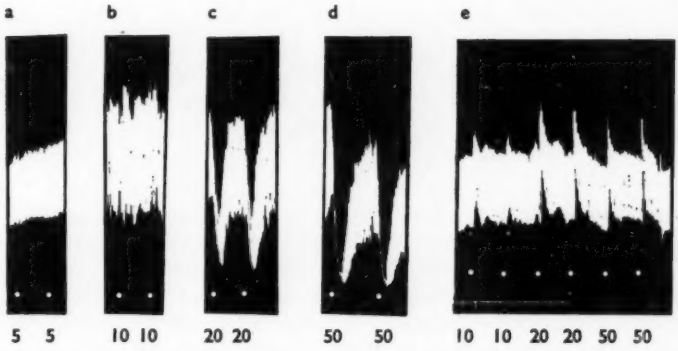

Fig. 5. Effect of rate of sympathetic nerve stimulation on response of isolated ileum before (*a–d*) and 20 min after guanethidine (*e*). Stimulation for 20 sec every 3 min with 2 msec pulses is indicated at the white dots; the frequency of stimulation in pulses/sec is given by the numeral. The time trace is in min.

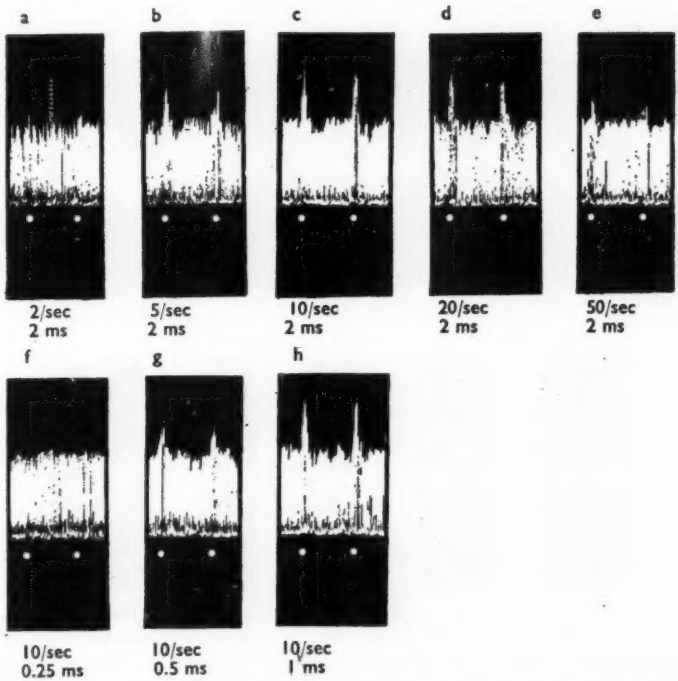

Fig. 6. Effect of varying frequency and pulse width on the motor response of ileum to sympathetic nerve stimulation after guanethidine (1 μg/ml.). Stimulation, at the white dots, was given for 25 sec every 4 min. The frequency in pulses/sec and the pulse width in msec (ms) is indicated in each panel.

stimulation was 10/sec. In our experiments with ileum the inhibitory response to sympathetic stimulation was well developed at 50/sec and less at lower frequencies (Fig. 5). The motor response to sympathetic stimulation after guanethidine was optimum at a lower rate; in Fig. 5 at 20/sec and in Fig. 6 at 10/sec. The pulse width of the square waves to give optimum motor responses was 1 to 2 msec (Fig. 6), which was the same pulse width which gave optimum inhibitor responses before guanethidine.

Transmural stimulation of segments of intestine with 2 msec, 8 V pulses at 50/sec gave an inhibition identical with that caused by sympathetic nerve stimulation. This inhibition was blocked by guanethidine. In order to produce a motor response by transmural stimulation it was necessary to increase either the frequency of stimulation or the width of the pulses. Thus 2 msec, 60 V pulses at 100/sec, or 5 msec, 60 V pulses at frequencies of 10 to 100/sec, were the minimum stimulus parameters necessary for a motor response with transmural stimulation; higher frequencies and longer pulse widths were more effective. Therefore it is unlikely that the motor response to sympathetic nerve stimulation after guanethidine was due to current spread from the electrodes.

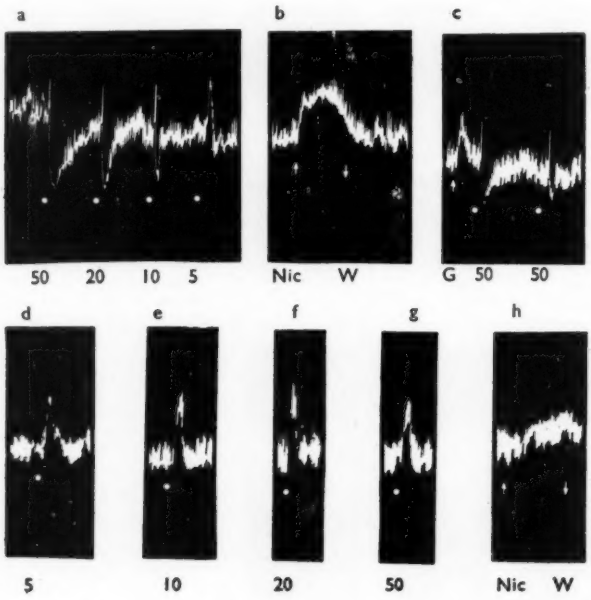

Fig. 7. Isolated rabbit colon. The periarterial sympathetic nerves were stimulated at the white dots with 2 msec pulses for 20 sec; the numerals refer to the frequency in pulses/sec. At Nic, nicotine bitartrate, 1 μg/ml., was added to the bath, and washed out at W. In panel *c*, guanethidine (G), 10 μg/ml., was added to the bath and remained in panels *d–h*, which were obtained 20 to 40 min later.

Region of intestine. Intestinal segments taken from duodenum and from various parts of the ileum and colon have given motor responses to sympathetic stimulation after guanethidine. In segments from the small intestine the inhibitor response was blocked by 1 μg/ml. guanethidine; segments from the large intestine were less sensitive and required 10 μg/ml. Fig. 7 is the record from a segment of colon. Stimulation at 5/sec before guanethidine resulted in a motor response; as the frequency of stimulation was increased the inhibitory component became more marked. After guanethidine stimulation at 5, 10, 20 and 50/sec produced approximately equal motor responses.

The effect of the age of rabbit on the responses. In the initial experiments it appeared that the motor response was more regularly observed in segments of ileum from young rabbits. In order to test whether age was a factor which affected the appearance of motor response to sympathetic stimulation, the observations recorded in Table 1 were made on ileal segments from litter-mate rabbits taken as close as possible to corresponding regions.

TABLE 1

GUANETHIDINE ON RESPONSE OF ILEUM TO SYMPATHETIC STIMULATION AT DIFFERENT AGES

Litter I				Litter II			
		Response to sympathetic stimulation				Response to sympathetic stimulation	
Age in days	Body wt. (kg)	Before guanethidine	After guanethidine	Age in days	Body wt. (kg)	Before guanethidine	After guanethidine
12	0·175	Motor response preceding inhibition	Motor response	12	0·155	Motor response preceding inhibition	Motor response
27	0·60	Motor response preceding inhibition	Motor response	27	0·45	Motor response preceding inhibition	Motor response
44	1·2	Motor response preceding inhibition	Motor response	44	0·7	Inhibition only	Motor response
61	1·6	Inhibition only	Motor response in ½ segments tested	61	1·1	Inhibition only	Motor response in ½ segments tested

In these experiments the response of segments of ileum was observed at various frequencies of stimulation. In the younger rabbits stimulation of the sympathetic at low frequencies (10 to 20/sec) produced an initial motor response followed by inhibition. With a higher frequency of stimulation (50/sec) a pure inhibitor response was obtained. After guanethidine the motor response was enhanced and the inhibition was abolished. In older rabbits inhibition only was seen at all frequencies. Finkleman (1930) described an initial motor response followed by inhibition after sympathetic stimulation of the nerves to the isolated rabbit intestine. He found

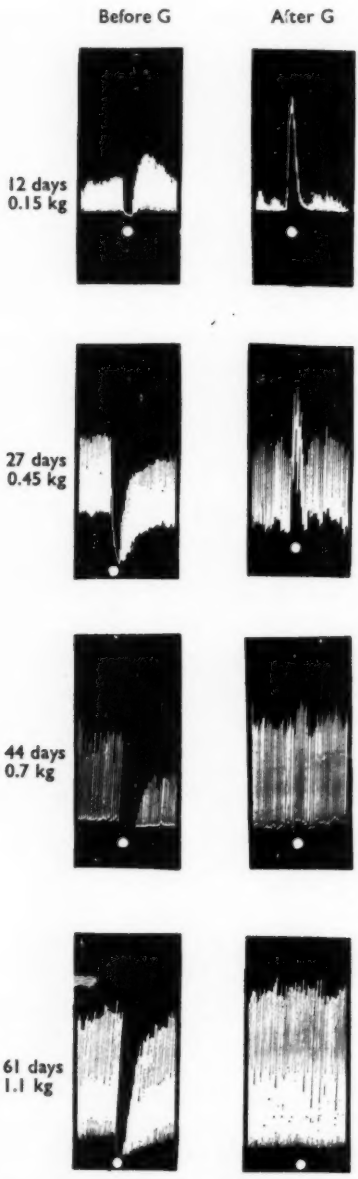

Fig. 8. Segments of isolated ileum from litter-mate rabbits of various ages and weights, indicated on the left. Sympathetic stimulation at the white dots was with 2 msec pulses at 50/sec for 20 sec. The left-hand column shows the responses before, and the right-hand column 20 to 30 min after, 1 μg/ml. guanethidine.

that these motor responses were seen only at low frequencies of stimulation and in the early part of the experiment, and were seen only in occasional rabbits. He made no comment on the age of the rabbits in which he saw a motor response.

Fig. 8 shows the responses of ileal segments of rabbits of various ages to sympathetic stimulation before and after the addition of guanethidine to the bath. In the 12-day-old rabbit the motor response is particularly well marked, but in the segment of ileum taken from a litter-mate killed at 61 days no motor response could be seen ; in this 61-day-old rabbit only one segment of three tested showed a motor response after guanethidine. In two litter-mates tested at intermediate ages the motor response was clear at 27 days and just detectable at 44 days.

In order to determine whether the increased motor responses in ileal segments from young rabbits was due to an increased sensitivity to acetylcholine, or to a decreased sensitivity to noradrenaline, experiments like that shown in Fig. 9 were

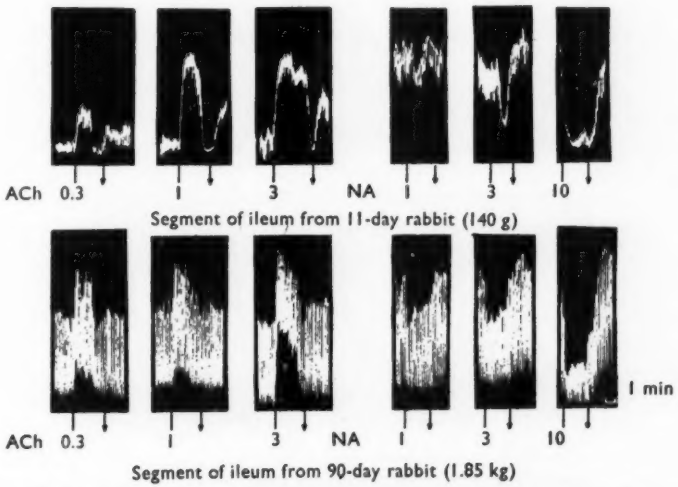

Fig. 9. Effect of acetylcholine (ACh) and noradrenaline (NA) on isolated ileum from young and adult rabbits. The figures beneath the vertical strokes indicate the dose of drug in μg in 70 ml. bath. The drugs were washed out at the arrow.

carried out. The nature of responses to both acetylcholine and noradrenaline were different in old and young rabbits. In young rabbits the amplitude of the rhythmic contractions was less and these drugs produced alterations in tone which exceeded in amplitude that of the rhythmic excursions. However, there was no age-dependent difference in sensitivity to either acetylcholine or noradrenaline.

Effect of degeneration of vagus nerves. The vagus nerves to the small intestine were degenerated either by cutting the right vagus in the neck or by cutting both vagi at the level of the abdominal portion of the oesophagus. Segments of jejunum were tested 8 to 20 days after nerve section. In 2 out of 5 rabbits, in which the

right vagus was sectioned in the neck, a motor response to sympathetic stimulation after guanethidine was seen in intestinal segments. In 2 out of 5 rabbits, in which both vagi were sectioned in the abdomen, motor responses of intestinal segments after guanethidine were convincingly demonstrated. Two other rabbits gave a slight indication of a motor response.

Xylocholine (TM10), bretylium and reserpine. In two experiments with xylocholine (5 μg/ml.) a motor response did not develop, and the inhibitor response to sympathetic stimulation was not completely blocked. In two experiments with bretylium (5 μg/ml.) the inhibitor response to sympathetic stimulation was com-

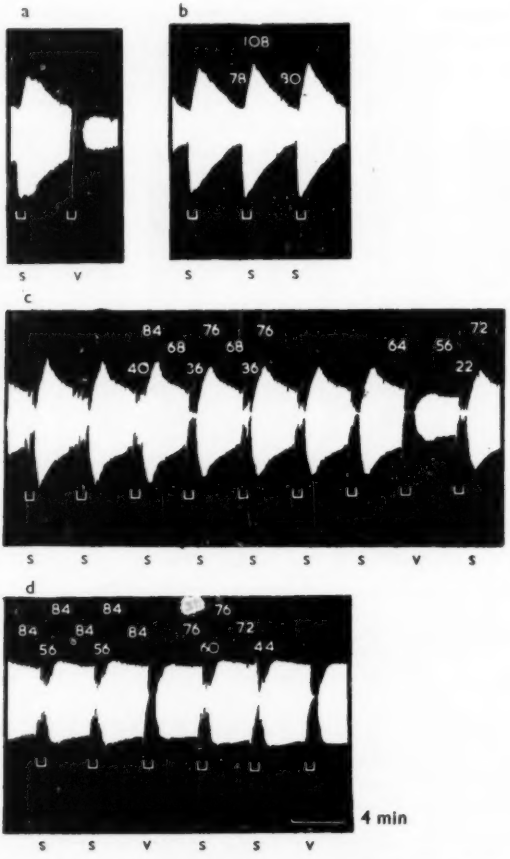

Fig. 10. Response of isolated cat atria to sympathetic stimulation at S and vagus stimulation at V. Stimulation, indicated by |__|, was with 2 msec pulses at 50/sec for 45 sec every 4 min. The numerals above the tracing refer to the rate of atrial contractions in beats/min. Control observations are shown in *a* and *b*; *c* is in the presence of 1 μg/ml. guanethidine, and *d*, 5 μg/ml. guanethidine.

pletely blocked, but no motor response was seen. A motor response to sympathetic stimulation was seen after pretreatment of rabbits with reserpine, thus confirming the observations of Gillespie & MacKenna (1961). When reserpine (1 μg/ml.) was added to the bath containing a normal segment of ileum, the inhibitor response was reduced after 3 to 4 hr but not abolished. A motor response was seen in 1 of 3 segments treated with reserpine in the bath. In 3 experiments with intestinal segments taken from rabbits previously injected daily with guanethidine, 10 mg/kg, for 4 days, the inhibitor response to sympathetic stimulation was no different from that of normal controls.

Cat atria. Responses of isolated cat atria to sympathetic and vagus nerve stimulation are shown in Fig. 10. When periods of sympathetic nerve stimulation were regularly repeated the pattern of the accelerator response and increase in amplitude of beating became constant and remained steady for long periods. After the addition of guanethidine (1 μg/ml.) to the bath the response to sympathetic nerve stimulation changed from uncomplicated increase in rate and amplitude to a mixed response consisting firstly of a decrease in rate and amplitude. This inhibitory phase, which resembled the response to submaximal vagus nerve stimulation, was followed by an increase in rate and amplitude which was similar to, but smaller than, the sympathetic response before guanethidine. After a larger dose of guanethidine (5 μg/ml.), only the first inhibitory phase of the response to sympathetic nerve stimulation was seen. At the same time the inhibition produced by vagal stimulation was less, and the record (Fig. 10d) shows that the responses to sympathetic nerve stimulation and vagus nerve stimulation are barely distinguishable.

DISCUSSION

The response of rabbit intestine to stimulation of extrinsic sympathetic and parasympathetic nerves has been recently studied and reviewed (Garry & Gillespie, 1955). It has generally been held that the occasional appearance of cholinergic responses to stimulation of sympathetic nerve trunks is due to parasympathetic fibres included in the sympathetic nerve trunk (Finkleman, 1930). Gillespie & MacKenna (1961) found that degeneration of the sacral parasympathetic nerves abolished the motor responses of the colon from the reserpinized rabbit to sympathetic stimulation. They suggested that reserpine might have the property of activating in some way the parasympathetic pathway, such that sympathetic nerve stimulation can then give rise to typically parasympathetic effects. We have three pieces of evidence that the parasympathomimetic effects that we have observed after stimulating the periarterial sympathetic nerve supply to the rabbit small intestine are not mediated entirely via parasympathetic nerves. Firstly, the motor response was not blocked by hexamethonium, although it was reduced in some experiments; secondly, the motor response was still present after section of the vagus nerves; and, thirdly, a motor response to sympathetic stimulation is more readily produced in young rabbits than in adults, and it is unlikely that the course followed by parasympathetic fibres should change during the first few months after birth. One possible explanation for the more pronounced motor response in young rabbits may be that the catecholamine content of the intestine is lower than in adults. In the adrenal glands from

several species the total catecholamine content increases with age (Hökfelt, 1951 ; West, Shepherd & Hunter, 1951). If this explanation is correct the motor response to sympathetic stimulation in intestine from young animals is due to cholinergic fibres whose presence is later concealed when greater stores of noradrenaline become laid down and the inhibitor response is consequently greater. The motor response of the intestine to sympathetic stimulation in reserpine-treated rabbits is presumably the reverse of this ; the stores of catecholamine are depleted, the inhibitor response less, and the motor response is thereby revealed.

Large doses of guanethidine have been shown to have the property, in common with reserpine, of depleting catecholamines from peripheral tissues (Bein, 1960 ; Cass, Kuntzman & Brodie, 1960). Maxwell *et al.* (1960) suggested that guanethidine acted by interfering either with the release, or the distribution subsequent to release, of transmitter substances from sympathetic nerve terminals, or in both these ways.

Cass, Kuntzman & Brodie (1960) showed that a single intravenous dose of 12.5 mg/kg of guanethidine depleted the noradrenaline in rabbit heart and spleen. More recently, Cass (personal communication) found that a single dose of 1 mg/kg of guanethidine did not reduce the noradrenaline content of rabbit tissues, but this dose of guanethidine is sufficient to abolish the usual response to sympathetic nerve stimulation *in situ*. In the organ bath 1 μg/ml. of guanethidine abolished the adrenergic response to sympathetic stimulation, but the noradrenaline content of segments of intestine taken from the organ bath at this time is the same as that of control segments (Cass, to be published).

The effect of guanethidine in altering the response to sympathetic nerve stimulation from an adrenergic to a cholinergic effect can be understood in the light of the hypothesis advanced by Burn & Rand (1959b, 1960a, b ; Burn, 1961) that the role of acetylcholine liberated from cholinergic sympathetic fibres is to release noradrenaline from a store in the vicinity of the nerve endings, so that nerve stimulation is ultimately adrenergic. We suggest that the action of guanethidine may be to prevent the release of noradrenaline by acetylcholine ; then the action of acetylcholine acting directly on muscarinic receptors is revealed.

In some patients treated with guanethidine hypotension is produced by exercise (Laurence & Rosenheim, 1960). This could be brought about if the effect of guanethidine were to abolish the adrenergic response to sympathetic nerve discharge and to replace it by a weak cholinergic response. McCubbin, Keneko & Page (1961), working with the dog perfused leg, found that guanethidine altered the response to sympathetic stimulation from vasoconstriction to dilatation which could be blocked by atropine.

The effect of guanethidine in altering the response of the cat isolated atria to sympathetic stimulation from tachycardia to bradycardia can explain the occurrence of bradycardia in patients treated with guanethidine (Page & Dustan, 1959). If during sympathetic blockade by guanethidine the sympathetic cholinergic nerves to the heart were incapable of releasing noradrenaline, then it is conceivable that the direct action of acetylcholine on the heart might be revealed.

We are grateful to the Wellcome Trust for the support of one of us (M. J. R.) and to Messrs May & Baker for giving the opportunity to M. D. D. to work on this subject.

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SOME EFFECTS OF A MONOAMINE OXIDASE INHIBITOR UPON CHANGES PRODUCED BY CENTRALLY ADMINISTERED AMINES

BY

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In cats treated with subcutaneous or intraperitoneal injections of the monoamine oxidase inhibitor phenylisopropylhydrazine, the effects of 5-hydroxytryptamine injected into the lateral cerebral ventricle were greatly intensified and prolonged; the effects of adrenaline were potentiated to a lesser extent. Those of noradrenaline, dopamine and tryptamine were not intensified and were prolonged to only a slight degree.

One of the problems involved in understanding the role of naturally occurring amines in the brain is concerned with their physiological inactivation. The enzyme monoamine oxidase has been thought to play the major role in this inactivation, but its physiological significance has become progressively more difficult to assess as evidence accumulates that alternative mechanisms exist for the metabolism of biogenic amines (Sjoerdsma, 1959). In addition, the substrate specificity of monoamine oxidase varies considerably according to the source of the enzyme (Hagen & Weiner, 1959). Drugs characterized as monoamine inhibitors are useful in counteracting certain depressive states, and this has helped to maintain strong interest in the role of this enzyme in central nervous system function. Doubt, however, has been expressed concerning the view that the clinical effects of these inhibitors result from their action on this enzyme (Rosenblum & Ferguson, 1960), as many other properties of the hydrazine monoamine oxidase inhibitors are known (Horita, 1961).

The behavioural effects of amines injected into the cerebral ventricles have been described by several investigators (Feldberg & Sherwood, 1954; Gaddum & Vogt, 1956; Bradley, 1958). A major advantage of the intraventricular method of administration is that it allows the direct observation of central effects of drugs without the complication of peripheral vascular effects.

In the present experiments, the effect of pretreatment with a hydrazine monoamine oxidase inhibitor (phenylisopropylhydrazine) was examined upon behavioural and other changes occurring after the intraventricular administration of catecholamines

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and indoleamines. In some experiments, the effect of 5-hydroxytryptamine was examined after pretreatment with reserpine.

METHODS

The amines were injected into the lateral cerebral ventricle of conscious cats through an indwelling Collison cannula. The method of implantation of the cannula under anaesthesia and aseptic conditions was that described by Feldberg & Sherwood (1953).

The amines were given in a volume of 0.25 ml. of saline solution. An interval of at least one week was allowed between each injection. The amines used were adrenaline and noradrenaline bitartrate, 3-hydroxytyramine (dopamine) hydrochloride, 5-hydroxytryptamine creatinine sulphate, and tryptamine hydrochloride. All doses refer to the free bases.

Phenylisopropylhydrazine (kindly supplied by Lakeside Laboratories, Milwaukee, U.S.A.) was given subcutaneously or intraperitoneally in daily doses of 2 mg/kg for up to three days. The amines were tested by the intraventricular route 2 to 4 or 24 hr after the last injection. Reserpine (Serpasil, Ciba) was given subcutaneously as a single injection; two cats received 0.05 mg/kg and two 0.2 mg/kg. The amines were tested by the intraventricular route 2 hr later.

RESULTS

Intraventricular injection of catecholamines and indoleamines. The intraventricular administration of 40 to 200 μg of adrenaline or noradrenaline resulted in drowsiness and disappearance of spontaneous activity. The cats would crouch in their cages, head nodding forward and eyes tending to close. Tachypnoea was regularly seen. Occasionally coarse or fine tremor was present in head and trunk. When pushed gently with a small rod, the cats became immediately wakeful. When they attempted to walk, ataxia was evident. Doses of over 100 μg often produced vomiting or retching. As shown in Table 1, the duration of the effects was slightly longer with adrenaline than with noradrenaline.

The effects of dopamine were similar to those of adrenaline and noradrenaline, but less intense. With doses of 200 μg the effects usually lasted 30 min to 2 hr (Table 1).

Intraventricular 5-hydroxytryptamine produced tachypnoea, loss of spontaneous activity, ataxia and often strong tremor. Drowsiness or vomiting was not observed. The duration of the effects of various doses (10 to 200 μg) is given in Table 1.

Tryptamine produced effects similar to, but less intense than, those produced by 5-hydroxytryptamine. Tremor was not seen. The effect of 200 μg lasted 30 min to 2 hr (Table 1).

Intraventricular injection of catecholamines and indoleamines after phenylisopropylhydrazine. Daily subcutaneous or intraperitoneal administration of 2 mg/kg of phenylisopropylhydrazine produced ataxia. This effect has been described by Spector, Shore & Brodie (1959) and by Vogt (1960). Ataxia appeared on the third day, but was not severe enough to prevent the cats from walking freely. Some animals developed profuse salivation, and appeared to be apprehensive and hyperexcitable. All effects disappeared within 24 hr after the last injection of phenylisopropylhydrazine.

TABLE 1

DURATION OF EFFECTS OF INTRAVENTRICULAR AMINES BEFORE AND 2 TO 4 HR, OR 24 HR,* AFTER THE LAST DAILY INJECTION OF 2 MG/KG PHENYLISOPROPYL-HYDRAZINE (PIH)

Compound and amount in μg given intraventricularly		Administration of PIH		Duration of effect in hr		
		Days	Route	Before	After PIH	
Adrenaline	40	1	Intraperitoneally	1	1	
	100	1		3	20	
	100	1		2	6	
	150	3	Subcutaneously	2	40	(death)
	200	3	Intraperitoneally	2	4	
	200*	3	Subcutaneously	3	24	
	200*	3		10	8	
	200*	3		4	10	
Noradrenaline	50	3		½	½	
	50	3		0	2	
	100	3		½	4	
	125	3		1	4	
	200*	3		3	6	
	200*	3		2	½	
	200*	3		½	4	
Dopamine	200	1	Intraperitoneally	0	3	
	200	1		2	6	
	200	1		½	1	
	200	1		2	4	
	200*	3	Subcutaneously	3	5	
	200*	3		1	1	
	200*	3		½	2	
5-Hydroxytryptamine	10	3		½	1	
	10	3		½	2	
	25	3		1	24	
	50	3		½	4	
	50	3		1	192	
	100	2		2	4	
	125	1	Intraperitoneally	0	1	
	200	1		2	72	(seizures)
	200	1		2	48	
	200	1		4	15	
	200	1		6	4	
	200*	3	Subcutaneously	4	48	(death)
	200*	3		2	24	
Tryptamine	200	3	Intraperitoneally	½	3	
	200	3		2	2	
	200	3		1	3	
	200	3	Subcutaneously	½	2	
	200	3		0	0	

After the treatment with phenylisopropylhydrazine the effects of intraventricular noradrenaline, dopamine and tryptamine lasted slightly longer (see Table 1). The sedative effect of noradrenaline appeared to be less pronounced, otherwise the effects of these amines were unchanged.

Treatment with the inhibitor intensified the ataxia and muscular weakness, but appeared to lessen the sedative effect of intraventricular adrenaline. The incidence of vomiting and retching was unchanged. The duration of the effects was prolonged to varying degrees. In three of the eight experiments shown in Table 1 the effects lasted for 20 to 40 hr. In these animals ataxia was severe and there was evidence of hind-limb weakness. The cats lay on their side or abdomen, and were unable

to crouch, sit or walk. One animal died. Severe effects of this kind have been described by Rothballer (1959) following intraventricular adrenaline alone when injected in a much larger dose (2 mg).

The treatment with phenylisopropylhydrazine intensified and prolonged the effects of intraventricular 5-hydroxytryptamine to a much greater extent than those of any of the other amines examined. Before the phenylisopropylhydrazine treatment the effects never lasted for more than a few hours; afterwards they persisted in half the experiments for 1 to 8 days (Table 1). During this time, the animals lay in their cages, unable to sit or stand or even crawl about. There was generalized muscular flaccidity although the patellar tendon reflex was unchanged. The animals were awake; their eyes were open and followed the observer. The only responses to gentle prodding were slight turning of the head and weak cries. The animals ate poorly and had to be fed with diluted milk. All except one animal recovered. Another animal developed recurrent seizures which began with twitching of the facial muscles and developed into brief generalized tonic-clonic convulsions. These ceased spontaneously after the postural effects had disappeared. There were short periods of twitching of the facial muscles in two other cats.

Intraventricular injection of 5-hydroxytryptamine after reserpine. The subcutaneous injection of 0.05 or 0.2 mg/kg of reserpine resulted in miosis and sedation as first described by Bein (1953). Miosis developed within 30 min and was followed by crouching and closing of the eyes. The animals were sluggish but could be induced to walk by gentle prodding. Fine tremors were noted at times. Signs of recovery were evident by 6 hr and full recovery occurred within 24 to 48 hr.

The reserpine treatment increased the tremor produced by intraventricular injection of 200 μg of 5-hydroxytryptamine. Two of the treated cats uttered intermittent cries during the first hour after the 5-hydroxytryptamine injection, but its other responses could not be distinguished from the general effects of subcutaneous reserpine; there was no evidence that they were changed or intensified.

DISCUSSION

The most striking observation was the intensification and prolongation of the effects of intraventricular 5-hydroxytryptamine seen in some of the cats pretreated with the monoamine oxidase inhibitor phenylisopropylhydrazine. This potentiation and prolongation cannot be explained by summation of the effects of the amines with those of phenylisopropylhydrazine and is likely to be the outcome of inhibition of monoamine oxidase activity. Potentiation of the responses to adrenaline also occurred after phenylisopropylhydrazine, but to a lesser extent. Intensification of the effects of noradrenaline, dopamine or tryptamine after phenylisopropylhydrazine did not occur, and their prolongation was so slight that it is not possible to decide whether this was due to a longer-lasting action of these amines or to summation of the effects with those of phenylisopropylhydrazine.

It is well known that tryptamine is less potent than 5-hydroxytryptamine on many smooth muscle preparations. This difference in potency also applies to the central effects observed after the intraventricular injection of these amines. A possible

explanation for the difference in potency of these two amines on peripheral structures has been proposed by Vane (1959). He has suggested that, since tryptamine is more fat-soluble than 5-hydroxytryptamine, it is likely to pass cellular diffusion barriers more readily than 5-hydroxytryptamine, and therefore would be more rapidly inactivated by intracellular amine oxidase. The present results do not support this explanation as applied to the central effects of these amines administered into the cerebral ventricles. If contact with amine oxidase were the decisive factor, the effects of tryptamine should have been potentiated to a greater extent after phenylisopropylhydrazine than those of 5-hydroxytryptamine. Since this did not occur in these experiments, inaccessibility to the action of monoamine oxidase is here not a consequence of the presence of the phenolic hydroxyl group in 5-hydroxytryptamine.

According to Goldberg (1959), the cardiovascular actions of dopamine and tryptamine are more significantly potentiated by monoamine oxidase inhibitors than those of adrenaline, noradrenaline or 5-hydroxytryptamine. In the present experiments the order in which phenylisopropylhydrazine potentiated the central effects of these amines was different: the effects of dopamine and tryptamine were hardly affected whereas those of adrenaline and 5-hydroxytryptamine were significantly increased. This apparent difference in potentiation of these amines suggests that the influence of enzyme inhibition on the effects of pharmacologically active substrates in the central nervous system differs from that in peripheral tissues.

Two alternatives may account for the fact that there was considerable individual variation in the effects of the intraventricular injection of the amines after pretreatment with phenylisopropylhydrazine. One is that inhibition of monoamine oxidase *in vivo* is incomplete and that the effects are dependent upon the degree of inhibition in each animal. The other is that the amines can be disposed of by means other than amine oxidase activity, such as simple diffusion away from their sites of action in the brain, their uptake by neighbouring tissues, or their metabolism via other pathways as exemplified by O-methylation of the catecholamines (Axelrod, 1959).

The author is indebted to Sir Charles Harington for hospitality, and to Professor W. Feldberg for his interest during these experiments.

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EFFECT OF RESERPINE ON GROWTH AND SEXUAL DEVELOPMENT OF CHICKENS

BY

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One-month-old male chickens were given injections of 1 mg, 2 mg and 4 mg/kg of reserpine. The injections were repeated at weekly intervals for three months. The chickens receiving reserpine grew at half the rate of untreated control chickens and failed to grow combs. At 4 months of age the testes of reserpine-treated chickens were only about one-tenth the weight of the testes of the control chickens and histologically they showed extreme hypoplasia of the seminiferous tubules. Two large doses of reserpine one month apart could cause atrophy of the testes of adult roosters.

In studies in which reserpine was used to deplete chicken adrenals of their adenine nucleotides and catechol amines (Burack, Weiner & Hagen, 1960) and in which roosters treated with stilboestrol served as experimental animals because they were cheap and readily available, the observation was made that control birds, which had not received reserpine, grew combs when kept for several months. On the other hand, those birds which had received a single injection of reserpine two or three months previously continued to look like capons. Thus, reserpine apparently prevented the appearance of this secondary sex characteristic (comb growth) which appeared in the control animals as the effect of the stilboestrol wore off.

The experiments reported in this paper were carried out in order to determine whether reserpine has an inhibitory effect on sexual development or can cause regression of gonadal activity in adult birds. The results of these experiments indicate that reserpine, injected once weekly in doses as low as 1 mg/kg, can inhibit both growth and sexual development of young male chickens, and that two large injections of reserpine with one month's interval between can cause atrophy of the testes of full-grown roosters. A preliminary report of this work has already been given (Hagen & Wallace, 1960).

METHODS

Effect of reserpine on maturation and on the development of secondary sex characteristics

One-month-old male (Sussex–Barred Rock) chickens were obtained from a commercial hatchery. They were divided into three groups of nine each and placed in three cages. In the first group six chickens received an intramuscular injection of reserpine 1 mg/kg body

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weight each week and three untreated chickens were kept in the same cage. In the second group six chickens received reserpine 2 mg/kg weekly and three untreated chickens were kept in the same cage. In the third group five chickens received 4 mg/kg reserpine weekly and four untreated chickens were kept in the same cage. The injected animals were identified by a band around the leg. After five weeks the control animals were so much more active that they were removed to separate cages lest their general ebullience might upset the less active reserpine-treated birds.

The chickens were fed a commercial growing-mash daily and excess wheat was always available on the floor of the cage. They were weighed at intervals of two weeks.

After ten weeks one chicken from each injected group and two of the control birds were killed and their testes, combs and wattles, thyroid glands and adrenal glands were removed and weighed. Their testes, thyroids and adrenals were also examined histologically.

Three and a half weeks later (13.5 weeks from the beginning of the experiments) all the chickens (now 17.5 weeks old) were killed and autopsied, the weights of their testes, combs and wattles determined and the testes examined histologically.

Effect of reserpine on adult roosters

Twenty White Leghorn roosters each of between 1.8 and 2.2 kg weight were divided into two groups of ten. Each of the members of one group received 30 mg reserpine in a volume of 0.3 ml. by intramuscular injection; each of the control group received 0.3 ml. of reserpine solvent (Burack, Weiner & Hagen, 1960). One month later each of the experimental group received a second injection of 25 mg of reserpine. Four of the reserpine-treated roosters died during the experiment. One of the remaining roosters was killed one week after the second injection and a control rooster killed at the same time. The remaining five were killed at the end of the second month after the initial injection and five control birds killed at the same time. The weights of the testes were recorded and portions of the testes fixed in formalin for histological investigation. The diet of these animals consisted of growing-mash and wheat *ad libitum*. However, food was removed from the cages of the control animals for three to four days following the administration of reserpine to the experimental birds, who showed no inclination to eat for several days after each injection. The food was returned to the control birds when the injected animals began eating again.

RESULTS

Effect of reserpine on body growth

The growth curves of the groups treated with 1 mg/kg/week and 4 mg/kg/week reserpine and of the control groups are shown in Fig. 1. Those birds receiving the lowest dose of reserpine (1 mg/kg) did not grow at a significantly faster rate than those receiving 4 mg/kg.

Effect of reserpine on comb growth

Four weeks after the beginning of the experiment combs had begun to develop on the control chickens but had failed to appear on those receiving reserpine. As the experiment progressed slight comb growth was evident on the injected birds, but the difference in comb growth between the controls and the treated animals became more marked (Fig. 2). The mean weight of the comb and wattles of all control animals was 9.6 ± 3.6 g. The mean weight of comb and wattles of all animals receiving reserpine was 1.2 ± 1.4 g (Table 1).

Effect of reserpine on testis development

With three exceptions, in animals receiving only 1 mg/kg reserpine, the testes of all animals receiving reserpine weighed less than 1 g. The mean weight of the

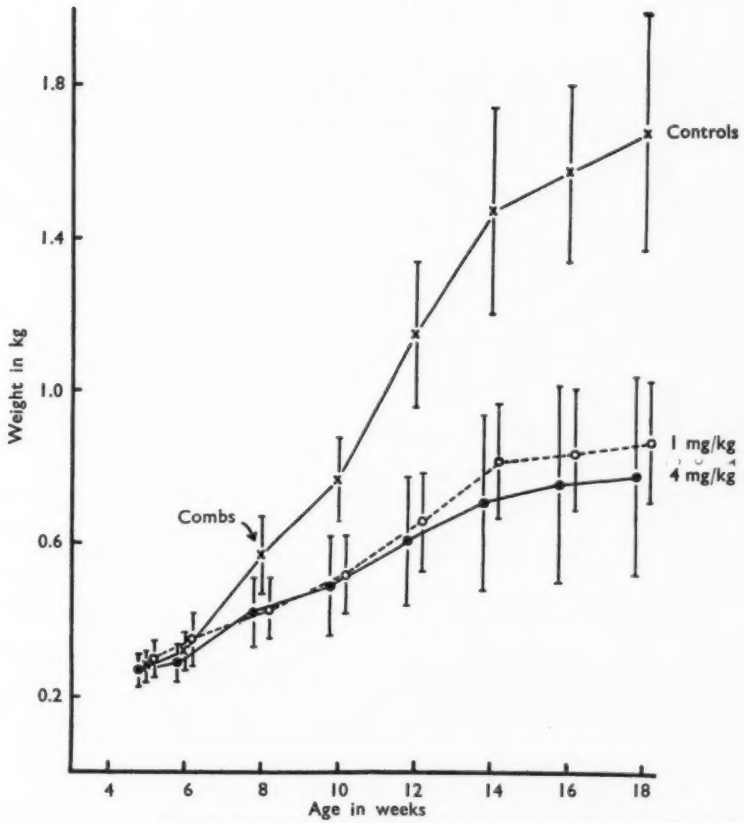

Fig. 1. Growth curves of male chickens showing the effect of weekly injections of reserpine begun at 5 weeks of age. The size of the weekly doses of reserpine is indicated on each graph. The time of appearance of definite combs is indicated on the graph. The vertical lines indicate standard deviations of the individual readings from the mean.

Fig. 2. Photograph of two of the chickens receiving reserpine 4 mg/kg weekly and of two control chickens all at 3 months of age.

TABLE 1

EFFECT OF RESERPINE ON WEIGHT OF CHICKENS AT 4 MONTHS OF AGE

Each chicken received weekly doses of reserpine since their fifth week of life

Dose of reserpine mg/kg	Number of animals	Mean weight (g) ± s.d.		
		Whole animal	Testes	Comb and wattles
0	10	1,682±316	8·10±2·20	9·60±3·60
1	6	870±160	1·42±1·83	2·00±2·00
2	6	960±172	0·26±0·28	0·98±0·55
4	5	780±264	0·43±0·57	0·42±0·26

control testes was 8.1±2.2 g; of the testes of reserpine-treated birds 0.72±0.52 g (Table 1). Histologically, the testes of all animals receiving 4 mg/kg of reserpine weekly showed extreme hypoplasia. The tubules were small in diameter and were lined almost invariably by a single epithelial layer. Most of the cells in the lining were Sertoli cells, although occasional spermatogonia were present. The interstitial tissue was very small in amount. In some testes very few Leydig cells could be seen, while in others they were fairly abundant; in no case did there appear to be hyperplasia of these elements. No spermatozoa were seen in the testes of this group (Figs. 3 and 4). Testes of those animals receiving reserpine 2 mg/kg weekly showed more variable degrees of atrophy. In most there was some reduction in

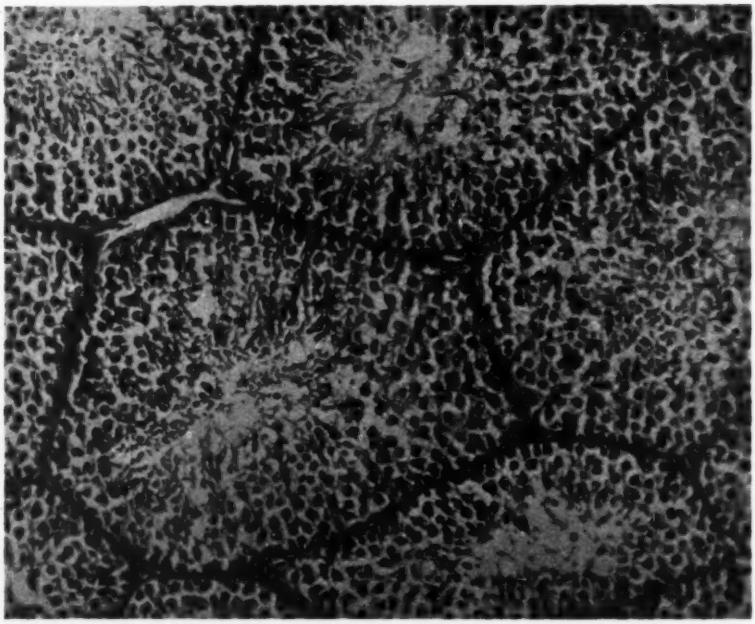

Fig. 3. Normal rooster testis showing all stages of spermatogenesis and tubule lumen filled with mature spermatozoa. The epithelium is many layers thick, and the interstitial tissue is relatively small in amount. (H. & E., ×250.)

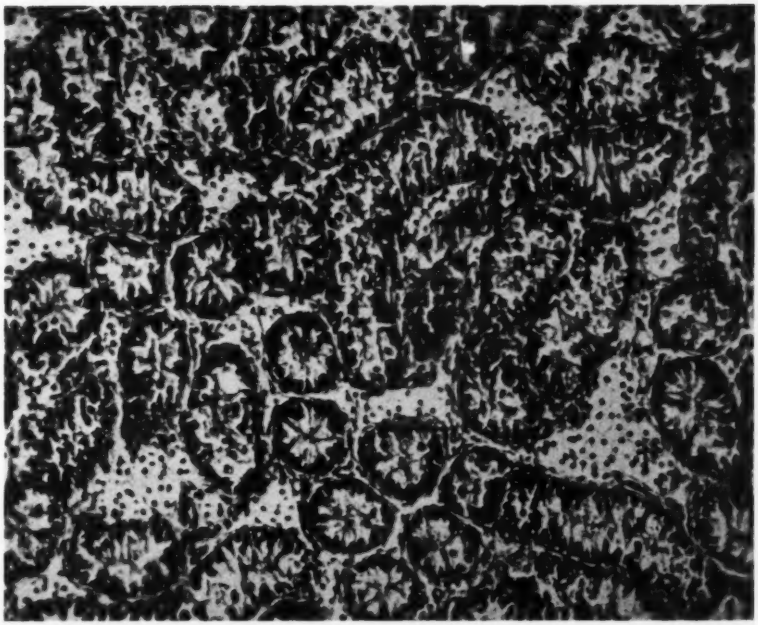

Fig. 4. Testis from animal receiving 4 mg/kg of reserpine weekly beginning at 1 month of age. There is an extreme degree of hypoplasia, the diameter of the tubules being about 15% of normal. The tubular epithelium consists for the most part of a single layer of Sertoli cells. No mature spermatozoa are present. Leydig cells are quite numerous in this section, but this was not the case with all the animals under treatment. (H. & E., ×250.)

tubular diameter, epithelial lining of about two cells in thickness, and a marked reduction in spermatogenesis. In the animals that received 1 mg/kg, the testes varied from completely normal to the picture of complete hypoplasia identical to that seen with 4 mg doses.

Effect of reserpine on thyroid and adrenal gland development

The weights of the thyroid and adrenal glands of the animals treated with reserpine did not differ significantly from those of the control roosters. They appeared normal on histological examination.

Effect of reserpine on testes of mature roosters

The weights of the testes of the six surviving adult roosters which received reserpine and of six control birds are shown in Table 2. The mean weight of the testes of the control birds was 9.3 ± 3.8 g and of the reserpine-treated birds 2.6 ± 1.7 g. Hypofunction of the testes was indicated in the course of the experiment by a marked reduction in size of the combs of some of the roosters receiving reserpine. However, some of the reserpine-treated animals did retain large combs throughout the experiment despite atrophy of the testes.

TABLE 2

BODY AND TESTICULAR WEIGHTS OF CONTROL ROOSTERS AND ROOSTERS TREATED WITH RESERPINE

* One control and one reserpine-treated rooster killed five weeks after the beginning of the experiment

Initial weight (kg)	Final weight (kg)	Testicular weight (g)
	Controls	
2·0	2·1	8·0
1·9*	1·8*	4·0*
2·2	2·3	12·0
2·3	2·2	12·0
2·1	2·1	14·0
2·2	2·1	6·0
	Reserpine treated	
2·0	1·9	0·7
1·9	1·9	4·0
2·0	1·9	3·0
2·2*	1·8*	1·0*
2·2	1·9	2·0
1·8	1·7	5·0

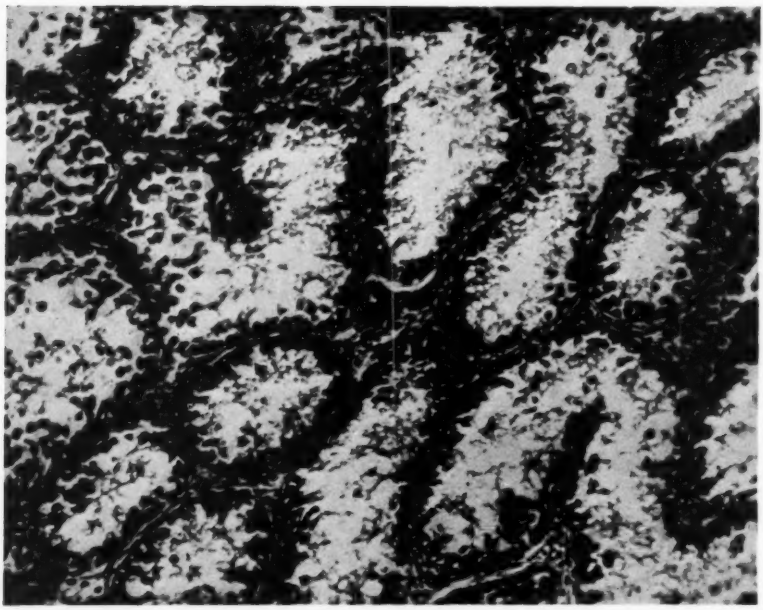

Fig. 5. Testis of a rooster receiving a total of 55 mg of reserpine in two doses. The tubular lumen is not as small as in the case of the testes of the immature animals treated, but the diameter is only 30% of normal. The epithelial lining is mainly a single layer of Sertoli cells, with occasional spermatogonia. No mature spermatozoa are present. In this section, the interstitial tissue is small in amount. (H. & E., ×250.)

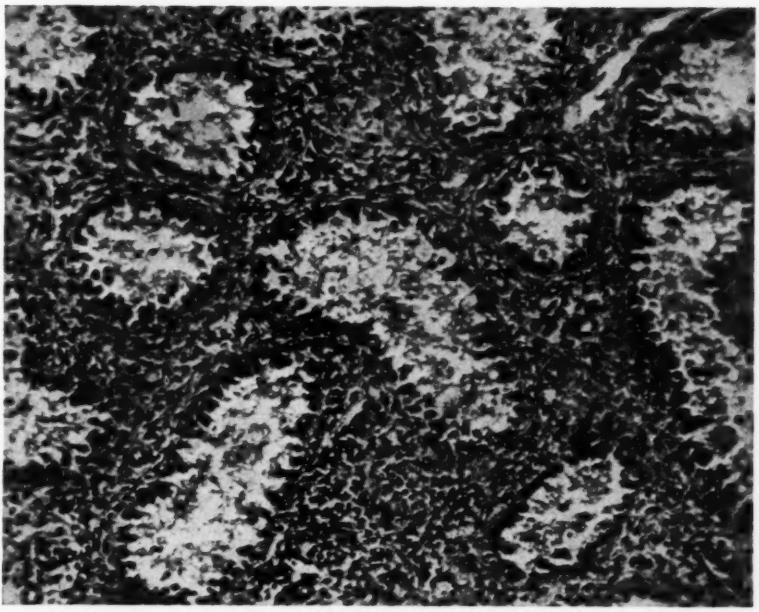

Fig. 6. Testis from another rooster receiving the same dose of reserpine as the animal whose testis is seen in Fig. 5. The appearance of the tubules is the same, but the interstitial tissue is much greater in amount. It appears to consist largely of fibrous tissue with chronic inflammation, and Leydig cells are not prominent. (H. & E., ×250.)

The histological appearance of the testes was essentially that of atrophy, similar to that described for the testes of roosters treated with female sex hormones (Emmens, 1939) or subjected to hypophysectomy (Nalbandov, Meyer & McShan, 1946). Animals given two doses of reserpine all showed profound testicular atrophy, although the reduction in tubule size was not as marked as that seen in the hypoplastic testes of the immature roosters. Spermatogenesis was totally absent ; tubules were lined with epithelial cells only one to three layers in depth, contrasted with a depth of about eight cells in the tubules of the control animals. The profound effect on spermatogenesis is contrary to reports of other workers who examined the testes of rats treated with reserpine (Albeaux-Fernet, Bellot, Breant, Bugard, Chabot, Deribreux, Gelinet & Romani, 1959). Most of the cells present were Sertoli cells. The interstitial tissue varied somewhat. In some animals it formed a narrow, acellular framework (see Fig. 5), while in others it was of considerable thickness (see Fig. 6). In the latter case the interstitial tissue was quite fibrous and contained abundant lymphocytes and monocytes. Leydig cells were not a prominent feature in any of the sections, and, judging by their lack of vacuolation, were not active. The rooster killed one week after the second dose of reserpine showed only a moderate degree of testicular atrophy, and spermatogenesis persisted at a reduced rate in both testes. Control animals' testes were entirely normal except for occasional small focal zones of atrophy, which comprised only a minute portion of the total area.

DISCUSSION

The results of the experiments described leave little doubt that reserpine can cause impairment of growth of young male chickens and slight loss of weight in adult roosters. In addition, reserpine can cause retardation of development of the testes so that at 18 weeks of age the testicular weights of those chickens receiving 2 or 4 mg/kg weekly were only one-sixteenth the weights of the testes of the controls. A dose of 1 mg/kg, which caused approximately the same reduction in weight, was less effective in inhibiting testicular growth, so that the weight of the testes was only about one-sixth the weight of the control testes.

In considering the site at which reserpine may be acting to cause testicular atrophy or hypoplasia several possibilities come to mind. A direct effect on the testes might either interfere with the normal growth of the testicular cells or antagonize the action of gonadotrophic hormone on the testes. On the other hand, it is possible that reserpine might act on the adenohypophysis to inhibit either the synthesis or secretion of gonadotrophins. Failure of these animals to grow suggests a possible inhibition of growth hormone secretion. In fact, an action of reserpine on the adenohypophysis to inhibit the secretion of not only growth hormone and gonadotrophic hormones but possibly also other hormones might be postulated. A more general action of reserpine on the hypophysis is suggested when one remembers that effects of reserpine on the secretion of other pituitary hormones are already known. Thus reserpine has been shown to reduce egg production and the fertility and hatchability of eggs of female chickens (Hewitt & Reynolds, 1957); it can cause release of corticotrophin and it can also prevent the secretion of corticotrophin in response to stress (Wells, Briggs & Munson, 1956). It can reduce thyroid activity and secretion of oxytocin in rats (Moon & Turner, 1959a and b). Although the evidence is insufficient to substantiate such a claim it may be justifiable to speculate that, in addition to its action to deplete nervous and other tissues of catechol amines (Holzbauer & Vogt, 1956) and 5-hydroxytryptamine (Paasonen and Vogt, 1956; Pletscher, Shore & Brodie, 1956), reserpine might well be able to deplete also the pituitary gland of the stores of several of its hormones.

In view of the other actions of reserpine on the endocrine glands discussed above, it would seem unlikely that the effects of reserpine on chicken testes are merely secondary to an inanition resulting from depression of appetite by this drug. Further evidence against such a mechanism is the much more marked effect of reserpine on the testes when given in a dose of 4 mg weekly than the dose of 1 mg weekly, although both doses of reserpine caused approximately the same retardation of growth.

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PHOSPHORYLPHOSPHATASE AND OXIMES

BY

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The effects of pralidoxime iodide, 1,1-trimethylenebis(4-formylpyridinium bromide) dioxime and diacetyl monoxime on the activity of phosphorylphosphatase prepared from pig kidney *in vitro* have been studied. It was found that these oximes, even at high concentrations, did not affect the activity of the enzyme.

Oximes have been shown to be effective antidotes against poisoning by organophosphates, including diisopropyl phosphorofluoridate (dyflos) (Kewitz, Wilson & Nachmansohn, 1956; Edery & Schatzberg-Porath, 1958; Hobbiger & Sadler, 1959). The most remarkable effect is their ability to reactivate phosphorylated acetyl cholinesterase; proteolytic enzymes with esterase activities, such as chymotrypsin (Green & Nicholls, 1959) and trypsin (Edery, to be published), can also be reactivated by high concentrations of the oximes.

Phosphorylphosphatase is an enzyme which appears to play an important role in the detoxification of dyflos in animals (Mounter, 1960) and possibly in human beings as well (Cohen & Warringa, 1954). Since there is no information about the effect of oximes on phosphorylphosphatase, it seemed of interest to examine this problem. It should be remembered that hydroxylamine, the parent compound of the oximes, has inhibitory effects on phosphorylphosphatase (Mounter, Floyd & Chanutin, 1953).

METHODS

A purified preparation of phosphorylphosphatase was obtained from pig kidney according to the method of Bergmann, Segal & Rimon (1957). The activity was determined by a manometric method described by the same authors.

The oximes used were: pralidoxime iodide (pyridine-2-aldoxime methiodide; 2-hydroxyiminomethyl-*N*-methylpyridinium iodide); 1,1-trimethylenebis(4-formylpyridinium bromide) dioxime {*N,N'*-trimethylenebis(4-hydroxyiminomethylpyridinium bromide)}; and diacetyl monoxime. A purified specimen of dyflos served as substrate for phosphorylphosphatase. Mn^{++} and Co^{++} were used as chlorides. Fresh solutions of all these substances were made in a buffer of the following composition: 0.1 M NaCl, 0.04 M $MgCl_2$, 0.026 M $NaHCO_3$ and 0.1% gelatin.

RESULTS

The oximes neither increased nor inhibited the activity of phosphorylphosphatase. The previous observations of Mounter *et al.* (1953), that Mn^{++} and Co^{++} ions

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potentiate phosphorylphosphatase activity, are confirmed; pralidoxime does not affect this potentiation. Table 1 summarizes most of the results obtained.

TABLE 1

EFFECTS OF OXIMES ON PHOSPHORYLPHOSPHATASE ACTIVITY

Phosphorylphosphatase incubated with pralidoxime (PAM), 1,1-trimethylenebis(4-formylpyridinium bromide) dioxime (TMB-4), diacetyl monoxime (DAM), Mn^{++} or Co^{++}, for 20 min in the side arm of a Warburg vessel before tipping into 5×10^{-3} M dyflos after equilibration of the system. Activity expressed as ml. CO_2/ml. undiluted enzyme/hr. Figures were corrected for non-enzymic hydrolysis and represent the mean of tw › separate experiments

Control activity	PAM 1×10^{-2}M	PAM 1×10^{-5}M	TMB-4 1×10^{-2}M	DAM 1×10^{-2}M	Mn^{++} 1×10^{-3}M	Mn^{++} 1×10^{-3}M and PAM 1×10^{-3}M	Co^{++} 1×10^{-3}M	Co^{++} 1×10^{-3}M and PAM 1×10^{-3}M
22·5	22·3	22·0	22·5	20·0	42·7	41·6	34·9	35·3

In other experiments phosphorylphosphatase was incubated with pralidoxime, 1,1-trimethylenebis(4-formylpyridinium bromide) dioxime, or diacetyl monoxime at a concentration of 1×10^{-1} M for 2 hr prior to the introduction of dyflos into the system. Under these conditions, too, phosphorylphosphatase activity was not affected.

DISCUSSION

The experiments show that oximes do not affect phosphorylphosphatase activity. It therefore seems reasonable to assume that they can be used in the treatment of dyflos poisoning without the risk of their interference with the natural enzymic process of detoxification.

Our thanks are due to Dr Sara Rimon (University of Tel Aviv) for her kind advice in the purification of the phosphorylphosphatase.

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EFFECT OF 5-HYDROXYTRYPTAMINE ON THE RESPIRATION OF EXCISED LAMELLIBRANCH GILL

BY

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5-Hydroxytryptamine, but not acetylcholine or catecholamines, stimulated the endogenous respiration of the excised gills of *Modiolus demissus* and *Mytilus edulis*. Respiratory stimulation by 5-hydroxytryptophan is presumed to have occurred only after it had been decarboxylated to 5-hydroxytryptamine. 2-Bromolysergic acid diethylamide inhibited the effect of 5-hydroxytryptamine, while lysergic acid diethylamide mimicked it. The glycogen that was degraded during incubation of the gill cannot account for all of the oxygen that was consumed, indicating that some other substrate within the gill was also oxidized. That the metabolic actions of 5-hydroxytryptamine may be related to its cilio-acceleratory activity is discussed.

There have been numerous observations to suggest that in many invertebrates 5-hydroxytryptamine acts as a neurohumoral agent (Welsh, 1955). Aiello (1957) and Gosselin & Ernst (1958) reported that 5-hydroxytryptamine caused a marked acceleration of the lateral cilia on the excised gill of *Mytilus edulis* and *Modiolus demissus*. Subsequent studies have revealed that the gill of *Mytilus* contains measurable quantities of 5-hydroxytryptamine and also the enzymes that are capable of producing (Milton & Gosselin, 1960) and of degrading (Blaschko & Milton, 1960) this amine. As a result of these findings Gosselin (1961) postulated that 5-hydroxytryptamine serves as a humoral regulator of the cilia on these gills.

Ciliary activity appears to be dependent upon the metabolism of the gill, since various metabolic inhibitors reduce ciliary activity (Gray, 1924 ; Weller & Ronkin, 1952 ; Aiello, 1960). The lack of information on the metabolism and the factors that control the metabolism of molluscan gill (Martin, 1961) prompted us to study the effects of 5-hydroxytryptamine on the metabolism of the excised gill in the hope of correlating the cilio-acceleratory actions of 5-hydroxytryptamine with some of its metabolic actions. In this paper we report the effects of 5-hydroxytryptamine and certain other neurohumoral agents on the respiration of the excised gills of two marine molluscs, *Mytilus edulis* and *Modiolus demissus*.

METHODS

Specimens of both *Modiolus demissus* from the Marine Biological Laboratories at Woods Hole and *Mytilus edulis* from Ipswich, Mass., were stored in moist containers at 4° C. Under

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these conditions *Modiolus* remained viable for more than two weeks. *Mytilus*, which are less hardy, were used within a week of shipping.

The entire gill was excised and immersed in artificial sea water of the following composition: NaCl, 423 mM ; KCl, 9 mM ; $CaCl_2$, 9.27 mM ; $MgCl_2$, 22.9 mM ; $MgSO_4$, 25.5 mM ; $NaHCO_3$, 2.15 mM ; final *p*H 7.75 to 7.85. After 20 to 30 min secreted mucus was removed, and each gill was cut into 2 or 3 pieces of equal size. One piece (70 to 100 mg wet weight) was placed in the main compartment of each Warburg flask. With sea water in the main compartment and agents to be added in the side arms, the final total volume of the reaction mixture was 2.0 ml. The centre well contained 0.2 ml. 2.5 N NaOH. The gas phase was oxygen and the bath temperature was 25° C. After a 10-min period of equilibration the manometer was read every 30 min for as long as 6 hr. Except in Tables 1 and 5, measured rates of oxygen uptake are reported as % of the control respiration. Specifically the "% of control respiration" equals 100 times the rate of oxygen consumption during the first hour after tipping divided by the rate for the 1 hr period before tipping. Gill protein was determined by a modification of the method of Gornall, Bardowill & David (1949) and was expressed as crystalline bovine albumin equivalent. Glycogen was determined by the anthrone method of Seifter, Dayton, Novic & Muntwyler (1950).

Drug concentrations are reported as the initial extracellular concentrations in contact with the gill tissue. The following drugs were used: 5-hydroxytryptamine creatinine sulphate, 5-hydroxytryptophan, acetylcholine iodide, (−)-adrenaline bitartrate, (−)-noradrenaline bitartrate, (±)-isoprenaline hydrochloride, eserine sulphate, lysergic acid diethylamide and 2-bromolysergic acid diethylamide.

RESULTS

Under the conditions of laboratory storage *Mytilus edulis* did not survive as well as *Modiolus demissus* ; the latter was therefore used for most of the experiments. Qualitatively similar results, however, were obtained with both species. When expressed as μl. of O_2 consumed per hr per mg of gill protein, the qO_2 in 50 separate determinations of *Modiolus* was 4.0 ± 0.09 (mean and standard error). Comparable values for 50 determinations in *Mytilus* were more than 50% higher (6.3 ± 0.17). Excised gill of both species continued to respire at a constant rate for at least 6 hr.

Effect of 5-hydroxytryptamine

As seen in Table 1, the rate of oxygen consumption by *Modiolus* gill in the presence of 5-hydroxytryptamine was significantly higher than the rate for the control

TABLE 1

EFFECT OF 5-HYDROXYTRYPTAMINE ON THE OXYGEN CONSUMPTION OF EXCISED GILL FROM *MODIOLUS DEMISSUS*

5-Hydroxytryptamine was added to the flasks at the start of the experiment, and the qO_2 was based upon a 2-hr incubation. qO_2 represents consumption of μl. O_2/hr/mg gill protein. Values are mean ± standard error of mean. *=Significantly different from control at 1% level

5-Hydroxytryptamine concentration	No. of expts.	qO_2
None (control)	9	4.0 ± 0.22
10^{-6} M	5	4.4 ± 0.55
10^{-5} M	6	5.8 ± 0.65*
10^{-4} M	12	7.8 ± 0.33*

gill. Because the respiration of the control gill was somewhat variable, stimulation by 5-hydroxytryptamine was more evident when each gill served as its own control. When measured in this manner a significant increase in the oxygen consumption

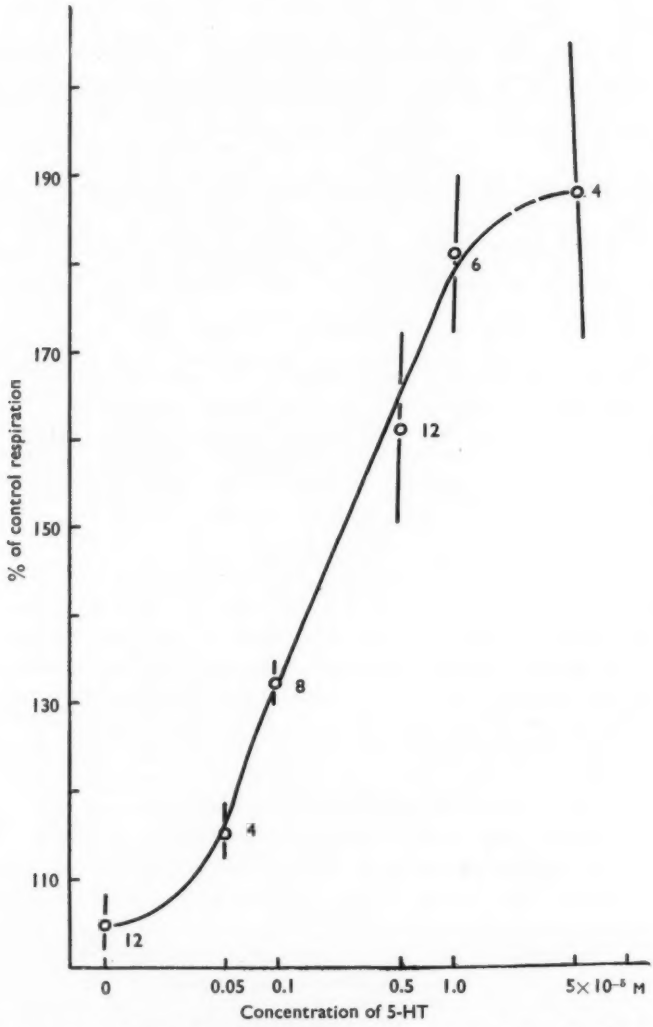

Fig. 1. Effect of 5-hydroxytryptamine on oxygen consumption of excised gill from *Modiolus demissus*. 5-Hydroxytryptamine was added after a 90-min control period. Each point represents the mean and the vertical line the standard error of the mean; the number beside each point denotes the number of experiments.

was observed at 10^{-6} M 5-hydroxytryptamine (Fig. 1). *Mytilus* gill was somewhat more sensitive to the action of 5-hydroxytryptamine; a significant rise in oxygen uptake was observed at 10^{-7} M. *Mytilus* is also more sensitive to the cilio-acceleratory actions of 5-hydroxytryptamine than is *Modiolus*; for dose-response curves on both

species see Gosselin (1961). The stimulatory effect of 5-hydroxytryptamine was rapid in onset; the maximal rate was noted in the first 30-min reading and was sustained for several hours. Neither control nor 5-hydroxytryptamine-stimulated respiration was affected by the addition of substrate amounts (0.01 M) of glutamate, glucose, succinate or α-ketoglutarate.

Effect of 5-hydroxytryptophan

It was reported previously (Milton & Gosselin, 1960) that 5-hydroxytryptophan produces prolonged stimulation of the beat frequency of lateral cilia on both *Mytilus* and *Modiolus* gill. When compared to the effects of 5-hydroxytryptamine, the response to 5-hydroxytryptophan was slower to develop and resistant to washing. Similar differences were encountered when the actions of 5-hydroxytryptophan on the respiration of the excised gill were studied. When 10^{-4} to 10^{-3} M 5-hydroxytryptophan was added, the oxygen uptake increased progressively with time; a maximal response was not reached until the second hour after addition of the compound (Table 2).

TABLE 2

EFFECT OF 5-HYDROXYTRYPTOPHAN ON OXYGEN CONSUMPTION OF EXCISED GILL FROM *MODIOLUS DEMISSUS*

Conc. of 5-hydroxy-tryptophan	No. of expts.	% of control respiration (mean±standard error)			
		1st hr	2nd hr	3rd hr	4th hr
None	7	102±2·5	106±2·1	105±3·1	107±4·5
10^{-5} M	8	106±3·3	111±2·8	109±3·8	112±7·5
10^{-4} M	8	142±13	162±12	161±11	152±15
10^{-3} M	8	149±4·8	169±10	165±13	146±33

Effect of other neurohormones: acetylcholine and catecholamines

Acetylcholine has been proposed as a cilio-regulatory hormone in the gill of *Mytilus edulis* (Bülbring, Burn & Shelley, 1953; Milton, 1959). The cilio-acceleratory action of this compound is much less pronounced and less sustained than that of 5-hydroxytryptamine (Gosselin, unpublished). When acetylcholine was added to the excised gill, either alone or in combination with eserine, there was no significant difference (at 1% level) from the control oxygen consumption (Table 3). Although data from only one concentration of acetylcholine are reported

TABLE 3

EFFECT OF ACETYLCHOLINE AND CATECHOLAMINES ON THE OXYGEN CONSUMPTION OF EXCISED GILL FROM *MODIOLUS DEMISSUS*

The concentration of each agent used was 10^{-4} M. Only two experiments were performed using isoprenaline; both results are given

Treatment	No. of expts.	% of control respiration (mean±standard error)
None	4	96±7·5
Eserine	4	100±4·2
Acetylcholine (ACh)	4	91±4·7
ACh+eserine	4	86±6·7
None	4	103±2·5
Adrenaline	4	107±6·4
Noradrenaline	4	116±4·5
Isoprenaline	2	107; 100

in Table 3, the results of experiments with other concentrations (10^{-7} to 10^{-4} M) were not different. Also summarized in Table 3 are the effects of three catecholamines. Although these compounds exert a pronounced calorigenic action and stimulate glycolysis in many vertebrate tissues, they had no prominent effect on the rate of oxygen uptake by these molluscan gills. The stimulatory effect of noradrenaline is significant at the 5% but not at the 1% level. However, even during the first experimental period, auto-oxidation (and perhaps enzymatic oxidation) of these amines occurred. If their oxidative destruction could have been inhibited by methods that did not interfere with tissue respiration, it is conceivable that different results might have been obtained.

Effect of 5-hydroxytryptamine antagonists

Attempts were made to block the respiratory stimulation seen after addition of 5-hydroxytryptamine with the 5-hydroxytryptamine antagonists, lysergic acid diethylamide and 2-bromolysergic acid diethylamide. Pronounced differences were observed between these two agents (Table 4). At 10^{-4} M lysergic acid diethylamide stimulated

TABLE 4

EFFECT OF 5-HYDROXYTRYPTAMINE ANTAGONISTS ON THE OXYGEN CONSUMPTION OF EXCISED GILL FROM *MODIOLUS DEMISSUS*

LSD=Lysergic acid diethylamide; BOL=2-bromolysergic acid diethylamide; 5-HT=5-hydroxytryptamine. *=Significantly different from control at 1% level

Treatment	No. of expts.	% of control respiration (mean±standard error)	
1. None	4	100±2·1	
2. LSD (10^{-4} M)	8	258±25*	
3. 5-HT (10^{-5} M)	4	209±28*	3 vs 4, $P=0{\cdot}2$
4. LSD+5-HT	4	272±41*	
1. None	6	101±5·3	
2. BOL (10^{-4} M)	6	113±4·7	
3. 5-HT (10^{-5} M)	6	211±13*	3 vs 4, $P<0{\cdot}01$
4. BOL+5-HT	6	141±6·3*	

markedly the oxygen consumption of gills ; smaller but significant effects were seen even at 10^{-6} M (not shown). Since a combination of 5-hydroxytryptamine (10^{-5} M) and lysergic acid diethylamide (10^{-4} M) resulted in a somewhat higher (not significant at the 1% level) rate of respiration than with either agent alone, no antagonism could be demonstrated. On the other hand, 2-bromolysergic acid diethylamide enhanced only slightly the gill respiration. When added along with 5-hydroxytryptamine, 2-bromolysergic acid diethylamide significantly reduced the stimulation produced by the former.

Effect of 5-hydroxytryptamine on glycogen breakdown

Since no exogenous substrate was required to maintain the respiration of excised gill, it was of interest to establish the nature of the endogenous substrates. The glycogen content of gill was determined before and after incubation in sea water. A representative series of analyses is shown in Table 5. Because the glycogen content of freshly excised gills was somewhat variable, each experiment was performed with the gills from one specimen. The concentration of glycogen was usually lower in *Mytilus* than in *Modiolus*, at least after the molluscs had been stored in

TABLE 5

EFFECT OF 5-HYDROXYTRYPTAMINE ON ENDOGENOUS CARBOHYDRATE METABOLISM OF EXCISED LAMELLIBRANCH GILL

Each experiment was performed on the gill from one specimen at 25° C in an atmosphere of 100% oxygen. All values are based on a 3-hr period of incubation and are adjusted to 100 mg weight of gill

	Modiolus		*Mytilus*	
	μl. O_2 consumed	Glycogen content (μg)	μl. O_2 consumed	Glycogen content (μg)
Expt. 1				
Not incubated	—	660	—	410
Sea water only	134	617	192	288
10^{-5} M 5-hydroxytryptamine in sea water	239	577	370	240
Expt. 2				
Not incubated	—	430	—	414
Sea water only	94	366	226	356
10^{-5} M 5-hydroxytryptamine in sea water	196	346	391	219

the laboratory. During incubation of the excised gill the glycogen content decreased; this reduction was accelerated by the addition of 5-hydroxytryptamine. Glycogen breakdown was somewhat more rapid in *Mytilus* than in *Modiolus*. Barely detectable amounts of lactic acid were observed in the incubation media at the end of these experiments.

DISCUSSION

It appears that there is a parallel between the 5-hydroxytryptamine-induced stimulation of oxygen consumption of excised lamellibranch gill and the stimulatory effects of this agent on the cilia of these gills. The respiratory response is not as sensitive to 5-hydroxytryptamine as is the beat frequency of the lateral cilia (Gosselin, 1961). However, other types of cilia on the same gills (for example, frontal and abfrontal cilia) are probably less sensitive to 5-hydroxytryptamine (Gosselin & O'Hara, 1961). Since oxygen uptake reflects the metabolism of all cells in the excised gill, including many that are not ciliated, it is not surprising that the correspondence between ciliary activity and gill oxygen consumption is not precise. Other agents that produce an acceleration of ciliary beat frequency, such as 5-hydroxytryptophan, lysergic acid diethylamide, and, to lesser extent, 2-bromolysergic acid diethylamide (Gosselin & Ernst, 1958), also produce an increase in the rate of oxygen uptake. Veratrine, which stimulates the beat frequency of *Mytilus* gill cilia, is also reported to increase the oxygen consumption of the excised gill (Aiello, 1960). Conversely, the catecholamines, which have equivocal effects on beat frequency (Bülbring *et al.*, 1953; Gosselin, unpublished data), have no pronounced effect on gill respiration. On the other hand, acetylcholine, which has been reported to have some excitatory activity on ciliary beat frequency (Bülbring *et al.*, 1953), does not augment the oxygen consumption.

Data on the respiratory actions of 5-hydroxytryptophan are consistent with the hypothesis (Milton & Gosselin, 1960) that it is the 5-hydroxytryptamine formed from 5-hydroxytryptophan and not the 5-hydroxytryptophan itself that is

responsible for the stimulatory effects of this compound on gill cilia. In contrast to the prompt responses to all other cilio-excitatory substances, 5-hydroxytryptophan produces a gradual rise in the rate of oxygen consumption over a period of 2 hr. As reported elsewhere (Milton & Gosselin, 1960), an increase in the endogenous 5-hydroxytryptamine content occurs when gills are incubated in 5-hydroxytryptophan solutions.

The effects of the 5-hydroxytryptamine antagonists are difficult to explain. Both 2-bromolysergic acid diethylamide and lysergic acid diethylamide were reported to stimulate cilia in low concentrations and to prevent the stimulation of cilia induced by 5-hydroxytryptamine in higher concentrations (Gosselin & Ernst, 1958). Studies of gill oxygen consumption led to distinctly different conclusions. Although 2-bromolysergic acid diethylamide displayed only slight metabolic activity alone, it effectively prevented the respiratory effects of 5-hydroxytryptamine. On the other hand, lysergic acid diethylamide had marked stimulatory effects, but did not prevent the actions of 5-hydroxytryptamine (Table 4). No explanation of these differences can be offered, but it is perhaps relevant that studies of ciliary activity were carried out with 5-hydroxytryptamine and its antagonists at concentrations 40-fold less than those specified in Table 4. It is also interesting to note that lysergic acid diethylamide mimicked, but 2-bromolysergic acid diethylamide blocked, the effects of 5-hydroxytryptamine on the carbohydrate metabolism of *Fasciola hepatica* (Mansour, 1959).

As in former studies of marine molluscan tissue (Glaister & Kerly, 1936), the addition of exogenous substrates failed to elevate the oxygen consumption of the excised tissues. Similarly, negative results were also obtained by Martin (1961) with tissues of cephalopods and snails. Undoubtedly one of the endogenous substrates is glycogen. Although glycogen breakdown was shown during aerobic incubation with and without added 5-hydroxytryptamine, the amount of glucose liberated, if completely oxidized, could account for only 30 to 50% of the oxygen consumed. Apparently the oxidation of some other substrates accounts for the remainder of the oxygen uptake. Further studies are required to establish the nature of these substrates.

The question of whether the effect of 5-hydroxytryptamine on the carbohydrate metabolism of the gills is due to a direct action on some metabolic enzyme system or whether it is secondary to increased ciliary activity is an important one. This report does not shed any light on this question. Efforts to stimulate the respiration of gill homogenates by the addition of 5-hydroxytryptamine have so far been unsuccessful. It would appear that the integrity of the cell must be preserved in order to show the metabolic effects of 5-hydroxytryptamine. Further work needs to be done on this point. In recent studies, Moore & Gosselin (1961) have found that 5-hydroxytryptamine markedly stimulates the anaerobic glycolysis of the excised gill. Since it has been reported that gill cilia do not beat under anaerobic conditions (Aiello, 1960), it would appear that 5-hydroxytryptamine exerts a direct effect on carbohydrate metabolism. We feel that the effect of 5-hydroxytryptamine on the carbohydrate metabolism may be responsible for both the increased oxygen consumption and accelerated ciliary activity that is observed after the addition of 5-hydroxytryptamine.

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INFLUENCE OF DICHLOROACETYLATION ON THE ANTIMICROBIAL ACTIVITY OF CHLORAMPHENICOL DERIVATIVES AND OF VARIOUS AMINES

BY

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The dichloroacetyl group, one of the functional groups responsible for the antibacterial activity of chloramphenicol, has been inserted into various amines, and the dichloroacetamido-derivatives obtained have been studied against *E. histolytica* EdM, *Candida albicans* ATCC 2091, *Saccharomyces cerevisiae* ATCC 7921, *Aspergillus niger* NRRL 3, *Trichomonas vaginalis* and *Mycobacterium tuberculosis* $H_{37}Rv$ and ATCC 607. Among the various chlorophenoxamide analogues, only *N*-(benzothiazol-2-ylmethyl)dichloro-*N*-2-hydroxyethylacetamide shows an activity, *in vitro* and *in vivo*, comparable with that of chlorophenoxamide. The dichloroacetyl group is essential for the high amoebicidal activity *in vivo* of chlorophenoxamide. The dichloroacetamidopropiophenones of known antifungal activity also show marked amoebicidal activity *in vitro*, but they appear to have a different mechanism of action from chlorophenoxamide since the dichloroacetyl group is not indispensable. The high antitubercular activity of some benzothiazole derivatives is not increased by dichloroacetylation.

Attempts at changing the chemical structure and configuration of chloramphenicol, leaving the dichloroacetyl group untouched, have not as yet led to the discovery of compounds with an antibacterial activity equal to that of the natural antibiotic.

Some derivatives, among the large number which have been synthesized and studied, do, however, show other biological activities which are slight or absent in chloramphenicol.

For instance, Long & Troutman (1951) have been able to demonstrate that α-dichloroacetamido-β-hydroxy-*p*-nitropropiophenone, whose structure differs from that of chloramphenicol by the transformation of the secondary alcohol group into a keto-group, with consequent disappearance of a centre of asymmetry, has an antifungal activity (particularly on *Candida albicans*) much greater than that of chloramphenicol itself.

Starting from these facts, we have studied the influence exerted by the dichloroacetyl group on the biological activity of various homogeneous classes of compounds. For this purpose we synthesized the substances classified in Tables 1 to 5.

METHODS

We determined the amoebicidal activity *in vitro* and *in vivo*, also the trichomonacidal, antifungal and antitubercular activities *in vitro*.

The *in vitro* amoebicidal activity was determined by performing microscopical examinations and subcultures of test-tubes with 4 ml. of the dilutions in Pavlova medium, 48 hr after seeding with 40,000 *E. histolytica* trophozoites, strain EdM (de Carneri, 1958a).

The *in vivo* antiamoebic activity was determined on young white rats by performing the examination of the caecum 5 days after injection of 250,000 trophozoites of the virulent Meah strain (Jones, 1946).

The trichomonacidal activity was tested on suspensions of 30,000 *Trichomonas vaginalis* in each ml. of CPLM medium (de Carneri, 1956a, b), reading the results after 24 hr at 37° C.

Antimycotic activity was tested on *Candida albicans* ATCC 2091, *Saccharomyces cerevisiae* ATCC 7921 and *Aspergillus niger* NRRL 3 by performing serial dilutions in Sabouraud's broth, seeding 10,000 micro-organisms/ml. and reading the results after 3 days at 27° C.

For determining the antitubercular activity, two strains of *Mycobacterium tuberculosis* were used: $H_{37}Rv$, of human origin, and ATCC 607, non-pathogenic and of rapid growth. Serial dilutions in Youmans medium were performed, each 4 ml. tube was seeded with 0.04 ml. of wet bacteria, and readings were taken after 14 days and 3 days at 37° C, respectively, the growth at the top and bottom of the tubes being observed.

RESULTS

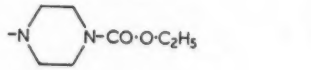
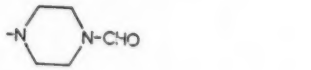
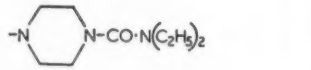
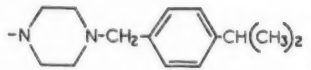
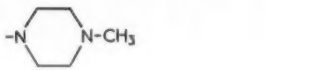
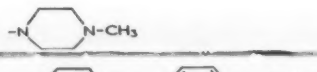
Derivatives of α-dichloroacetamidopropiophenone

Table 1 gives some basic derivatives of α-dichloroacetamidopropiophenone which were new compounds; these were obtained by a procedure already described (Alberti, 1956) consisting of the addition of secondary amines to α-dichloroacetamidoacrylophenones. By this method we prepared a series of substituted piperazines (compounds 1 to 7) and aziridines (compounds 8 to 11). Other compounds were obtained by acid hydrolysis of the aziridine derivatives (compounds 12 and 13) and by addition of isoniazid (isonicotinohydrazide) (compound 14). These propiophenone derivatives, and βp-dichloro-α-dichloroacetamidopropiophenone (compound 15), have considerable activity not only against several fungi (especially *Candida albicans*) but also against *Entamoeba histolytica in vitro* (de Carneri, 1958a, b).

It is well known that the antiamoebic activity of a compound can be exerted either directly on the parasite or through destruction of the bacterial flora on which the amoebae themselves live; but these compounds, whose structure is very similar to that of chloramphenicol, act directly on the parasite. This is shown clearly by their rapidity of action, the amoebae being killed within 1 hr after exposure to the lower effective doses. In addition, a direct amoebicidal action of a chloramphenicol analogue is also demonstrated by the fact that the L-*threo* isomer of chloramphenicol, which has no antibacterial activity, has the same amoebicidal activity as the natural antibiotic (de Carneri, 1956a). Except for compounds 10, 12 and 13, which are only slightly active, the new compounds listed in Table 1 show an amoebicidal activity of about 10 μg/ml. *in vitro*, while *in vivo* (in white rats) they do not display any activity. Studies now in progress show that the amoebicidal activity of compound 15 and of the corresponding acetyl derivatives is identical (10 μg/ml.).

TABLE 1

MINIMAL CONCENTRATIONS (μG/ML.) OF DICHLOROACETAMIDOPROPIOPHENONES OF THE GENERAL FORMULA $R.CO.CH(NH.CO.CHCl_2).CH_2.R'$ REQUIRED FOR COMPLETE GROWTH INHIBITION OF A SERIES OF MICRO-ORGANISMS

Com. pound no.	R	R′	*Entamoeba histolytica* EdM	*Candida albicans* ATCC 2091	*Sacchar. cerevisiae* ATCC 7921	*Asperg. niger* NRRL 3	*Trichom. vagin.*	*Myco. tuberculosis* $H_{37}Rv$	*Myco. tuberculosis* ATCC 607
1	$-NO_2.C_6H_4-$		12·3	7·8	15·6	31·2	250	15·6	62·5
2	$p-NO_2.C_6H_4-$		8·2	31·2	31·2	125	500	7·8	125
3	$p-NO_2.C_6H_4-$		8·2	31·2	62·5	250	250	7·8	125
4	$p-NO_2.C_6H_4-$		12·3	31·2	31·2	125	31·2	1·9	15·6
5	$p-Cl.C_6H_4-$		—	31·2	125	>125	—	—	—
6	C_6H_5-		12·3	11·8	31·2	125	500	1·9	15·6
7	$p-Cl.C_6H_4-$		—	15·6	15·6	31·2	—	1·9	15·6

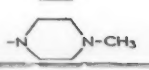
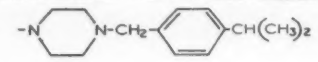
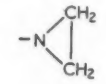
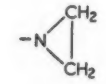
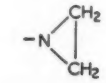
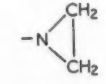
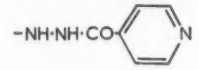
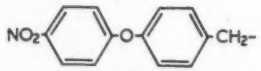
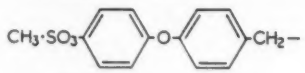
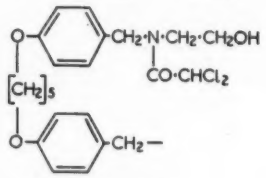
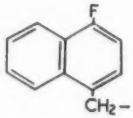
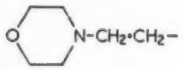
6	C_6H_5–		12·3	11·8	31·2	125	500	1·9	15·6
7	*p*-Cl.C_6H_4–		—	15·6	15·6	31·2	—	1·9	15·6
8	C_6H_5–		12·3	23·5	46·8	>125	500	0·9	15·6
9	*p*-$CH_3.SO_2.C_6H_4$–		12·3	125	>500	~500	250	—	—
10	*p*-$NO_2.C_6H_4$–		24·6	15·6	15·6	62·5	125	7·8	31·2
11	*p*-Cl.C_6H_4–		12	15·6	31·2	>500	125	3·9	15·6
12	*p*-Cl.C_6H_4–	–NH.CH_2.CH_2Cl	111	250	>500	>500	>500	31·2	125
13	*p*-Cl.C_6H_4–	–NH.CH_2.CH_2.O.SO_3H	12·3	15·6	15·6	62·5	125	15·6	62·5
14	*p*-$NO_2.C_6H_4$–		37	7·8	62·5	125	500	0·9	31·2
15	*p*-Cl.C_6H_4–	Cl	10	8	16	16	125	8	8

TABLE 2

MINIMAL CONCENTRATIONS (μG/ML.) OF ACETAMIDES WITH THE GENERAL FORMULA $R.N(CO.R'').CH_2.R'$ REQUIRED FOR COMPLETED GROWTH INHIBITION OF A SERIES OF MICRO-ORGANISMS

Compound no.	R	R′	R″	*Entamoeba histolytica* EdM	*Candida albicans* ATCC 2091	*Sacchar. cerevisiae* ATCC 7921	*Asperg. niger* NRRL 3	*Trichom. vagin.*	*Myco. tuberc.* $H_{37}Rv$	*Myco. tuberc.* ATCC 607
16		$-CH_2.O.CO.NH.CONH_2$	$-CHCl_2$	0·6	>500	>500	>500	>500	125	>500
17		$-CH_2OH$	$-CHCl_2$	37	>500	>500	>500	>500	250	500
18		$-CH_2OH$	$-CHCl_2$	>75	>500	>500	>500	>500	125	250
19	$CH_3-[CH_2]_{13}.CH_2-$	$-CH_2OH$	$-CHCl_2$	74	>500	250	>500	125	2·9	15·6
20		$-CH_2OH$	$-CHCl_2$	666	>500	>500	>500	>500	>500	125
21		$-CH_2OH$	$-CHCl_2$	>666	>500	>500	>500	>500	>500	>500
22		$-CH_2OH$	$-CHCl_2$	2·7	—	—	—	—	—	—

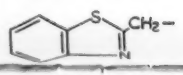
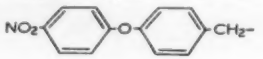
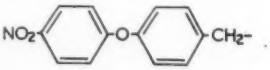
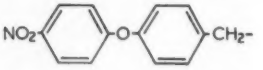
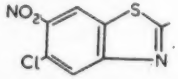
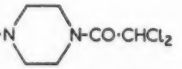
22		$-CH_2OH$	$-CHCl_2$	2·7	—	—	—	—	—	—
23		$-CH_2OH$	$-CHBr_2$	0·8	—	—	—	—	—	—
24		$-CH_2OH$	$-CH_3$	333	>500	>500	>500	>500	125	500
25		$-CH_2OH$	$-CHCl_2$	0·6	>500	>500	>500	>1,000	>500	>500
26	*p*-$HO.C_6H_4$–	$-CH_2OH$	$-CHCl_2$	666	—	—	—	>500	—	—
27	*p*-$Cl.C_6H_4$–	$-CH_2OH$	$-CHCl_2$	666	>500	>500	>500	>500	250	125
28		–H	$-CHCl_2$	666	—	—	—	>500	—	—
29	*p*-$HO.C_6H_4$–	–H	$-CH_3$	>666	>500	>500	>500	>500	>500	>500

TABLE 3

MINIMAL CONCENTRATIONS (μG/ML.) OF PIPERAZINES WITH THE GENERAL FORMULA  REQUIRED FOR COMPLETE GROWTH INHIBITION OF A SERIES OF MICRO-ORGANISMS

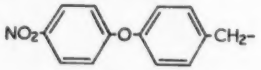
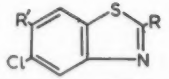
Compound no.	R	*Entamoeba histolytica* EdM	*Candida albicans* ATCC 2091	*Sacchar. cerevisiae* ATCC 7921	*Asperg. niger* NRRL 3	*Trichom. vagin.*	*Myco. tuberculosis* $H_{37}Rv$	*Myco. tuberculosis* ATCC 607
30	$C_6H_5.CH_2.CH_2$–	666	>500	>500	>500	>500	~125	31·2
31	*p*-$(CH_3)_2CH.C_6H_4.CH_2$–	111	>500	>500	>500	>500	62·5	>500
32		222	—	—	—	>500	—	—
33	$CH_3.[CH_2]_{10}.CH_2$–	4·1	>500	>500	>500	>500	0·4	250
34	*p*-$Cl.C_6H_4.CH_2$–	0·3	>500	>500	>500	—	—	—
35	$CH_3.CH_2.CH_2.CH_2$–	222	>500	>500	>500	>500	>500	>500

TABLE 4

MINIMAL CONCENTRATIONS (μG/ML.) OF BENZOTHIAZOLES OF THE GENERAL FORMULA



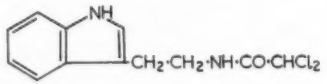
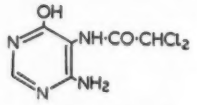
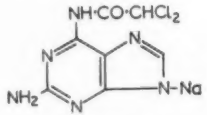
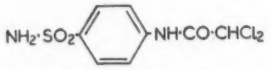
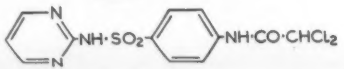
REQUIRED FOR COMPLETE GROWTH INHIBITION *MYCO. TUBERC.*

* Results of a subculture test. In the original test-tubes, a growth on the bottom was repeatedly observed only at 62·5 μg/ml.; the inhibition was complete at higher dilutions of the substance, up to 0·05 μg/ml.; full growth occurs at 0·01 μg/ml.

Compound no.	R	R′	*Myco. tuberc.* $H_{37}Rv$	*Myco. tuberc.* ATCC 607	Compound no.	R	R′	*Myco. tuberc.* $H_{37}Rv$	*Myco. tuberc.* ATCC 607	Compound no.	R	R′	*Myco. tuberc.* $H_{37}Rv$	*Myco. tuberc.* ATCC 607
36	$-SCH_3$	$-NH_2$	0·06	>500	41	$-SCH_3$	$-NH.CO.CH_3$	>500	>500	46	$-SCH_3$	$-NH.CO.CHCl_2$	>500	>500
37	$-SC_2H_5$	$-NH_2$	0·04	3·9	42	$-SC_2H_5$	$-NH.CO.CH_3$	0·1*	93·7	47	$-SC_2H_5$	$-NH.CO.CHCl_2$	0·1*	>125
38	$-NH_2$	$-NH_2$	>500	125	43	$-NH.CO.CH_3$	$-NH.CO.CH_3$	250	>500	48	$-NH.CO.CHCl_2$	$-NH.CO.CHCl_2$	15·6	>500
39	$-NH_2$	$-NO_2$	125	250	44	$-NH.CO.CH_3$	$-NO_2$	~250	>500	49	$-NH.CO.CHCl_2$	$-NO_2$	31·2	31·2
40	$-SH$	$-NH_2$	~500	~500	45	$-SH$	$-NH.CO.CH_3$	~500	>500	50	$-SH$	$-NH.CO.CHCl_2$	62·5	62·5

TABLE 5

MINIMAL CONCENTRATIONS (μG/ML.) OF A SERIES OF DICHLOROACETAMIDES REQUIRED FOR COMPLETE GROWTH INHIBITION OF A SERIES OF MICRO-ORGANISMS

Compound no.	Formula	*Entamoeba histolytica* EdM	*Candida albicans* ATCC 2091	*Sacchar. cerevisiae* ATCC 7921	*Asperg. niger* NRRL 3	*Trichom. vagin.*	*Myco. tuberculosis* $H_{37}Rv$	*Myco. tuberculosis* ATCC 607
51		250	>500	>500	>500	>500	5·8	>500
52		666	>500	>500	>500	>500	>500	>500
53		60	>500	>500	>500	>500	>500	>500
54		>600	>500	>500	>500	>1,000	125	>500
55		>600	>500	>500	>500	>1,000	125	>500

With regard to the antifungal activity, some compounds of Table 1 are completely inactive (9 and 12), while others show marked activity. Among these, compound 10 has been tested as an ointment in clinical practice, and has shown activity against fungal infections, particularly moniliasis. However, it decomposes slowly with time, turning red and losing part of its activity.

Compound 4 shows the most pronounced trichomonacidal activity of this series (31.2 μg/ml.).

Compound 14, an isoniazid derivative, shows, as expected, marked activity against *Myco. tuberculosis.*

Derivatives of chlorophenoxamide and diloxanide

Table 2 contains some new dichloroacetamido-derivatives with structures similar to those of dichloro-*N*-(2-hydroxyethyl)-*N*-[*p*-(*p*-nitrophenoxy)benzyl]acetamide (chlorophenoxamide) (de Carneri, Coppi, Almirante & Logemann, 1960), dichloro-*N*-(2,4-dichlorobenzyl)-*N*-(2-hydroxyethyl)acetamide (chlorbetamide) (Surrey, 1954) and dichloro-*N*-*p*-hydroxyphenyl-*N*-methylacetamide (diloxanide) (Bristow, Oxley, Williams & Woolfe, 1956). These three substances, whose extremely high activity *in vitro* and *in vivo* on *E. histolytica* have been shown by us and by others to exert a highly specific action on *Entamoeba,* are free from any antibacterial activity (de Carneri *et al.,* 1960 ; de Carneri, 1959): unlike the propiophenones previously mentioned, they exert not only an activity *in vitro* but also *in vivo* (de Carneri & Almirante, 1960).

Among the derivatives of Table 2, only *N*-(benzothiazol-2-ylmethyl)dichloro-*N*-2-hydroxyethylacetamide (compound 22) displays an *in vitro* and *in vivo* activity comparable with that of chlorophenoxamide, and this shows the importance of the diphenyl ether linkage to which the dichloroacetamido-group is attached (Logemann, Almirante & de Carneri, 1958 ; and Almirante, de Carneri, Coppi & Logemann, 1960).

The allophanic acid derivative corresponding to chlorophenoxamide itself (compound 16) is active *in vitro,* while it is completely inactive *in vivo.* The replacement of the chlorine atoms by bromine atoms (compound 23) also gives rise to a product with equal amoebicidal activity *in vitro,* but with less activity *in vivo* than chlorophenoxamide.

Compound 24, where the acetyl group has replaced the dichloroacetyl group, is also inactive. The determining influence of the dichloroacetyl group in this type of compound has already been shown in chlorbetamide (Surrey, 1954 ; and Surrey & Rukwid, 1955), where the replacement of this group by the monochloro- and trichloro-acetyl groups led to a reduction in activity.

Of the compounds similar to diloxanide (compounds 26 to 29), the benzothiazolyl derivative (compound 28) is completely inactive. Compounds 26 and 27, having a side-chain in which the methyl group has been replaced by the 2-hydroxyethyl group characteristic of chlorophenoxamide, are also inactive. In these derivatives also the dichloroacetyl group is determinant: replacement with the acetyl group cancels the activity (compound 29).

Compounds shown in Table 2 do not present other particular activities apart from the antitubercular effect of compound 19 which may be due to the presence in this compound of a long aliphatic chain.

N-*Dichloroacetamidopiperazine derivatives*

Table 3 consists of a series of *N*-dichloroacetamidopiperazine derivatives. While compounds 33 and 34 show a high activity *in vitro* on *E. histolytica*, they are inactive in the rats, even at a dosage of 60 mg/kg given *per os* or *per anum* (de Carneri *et al.*, 1960). Compound 33 is slightly active against strain ATCC 607, but shows a strong action against *Myco. tuberculosis* strain $H_{37}Rv$, probably due to the long aliphatic chain of this compound.

Benzothiazole derivatives

Table 4 contains some new benzothiazole derivatives. Our previous studies (Logemann, Galimberti, de Carneri & Coppi, 1960) showed the marked activity of some benzothiazole derivatives against *Myco. tuberculosis*. 6-Amino-5-chloro-2-ethylthiobenzothiazole, for instance, has an antitubercular activity *in vitro* equal to that of isoniazid.

The difference in the antitubercular activity of the amino-derivatives and the corresponding acetyl and dichloroacetyl derivatives of benzothiazole is clearly demonstrated by Table 4. Acetylation of the two most active compounds (36 and 37) leads to distinct reduction of the activity (compounds 41 and 42). Dichloroacetylation of compounds 38 and 39, which are in themselves only slightly active, leads to a marked increase in activity (compounds 48 and 49), but not enough to make these compounds of clinical value.

In contrast, acetylation of compounds 38 and 39 did not lead to any improvement in activity (compounds 43 and 44). None of the compounds included in Table 4 displayed any remarkable activity on *E. histolytica*.

Various compounds

Table 5 shows products obtained by dichloroacetylation of heterocyclic amines of different structures; two dichloroacetylated sulphonamides are also included. These compounds show no special activity.

DISCUSSION

An overall examination of the new compounds in Tables 1 to 5 shows that it is difficult to establish any definite relation between biological activity and presence of the dichloroacetyl group in the compounds as a whole; the presence of the dichloracetyl group determines the activity in only a few cases. It is therefore preferable to compare the activity of various compounds by examining their behaviour, within each single homogeneous class.

The dichloroacetamidopropiophenones, whose antifungal activity is already known, show high amoebicidal activity *in vitro* (Logemann, Lauria, Tosolini & de Carneri, 1958). They have a direct action on amoebae, although they are also antibacterial and can thus act on the parasite by an indirect mechanism through

destruction of the associated flora. Some are also slightly active on *Trichomonas vaginalis*. The dichloroacetyl group is not indispensable for amoebicidal activity, as it can be replaced by other acyl groups without the compounds losing their activity.

In contrast, the dichloroacetamido-group determines the activity of chlorophenoxamide derivtaives. The amoebicidal activity on trophozoites of *Entamoeba* is specific. The group to which the dichloroacetamido-group is linked is also of great importance in these compounds. For instance, in chlorophenoxamide the dichloroacetamido-group is attached to the diphenyl ether group which can be considered most suitable from this point of view, because the chemical stability and low absorption of its derivatives allow it to achieve high concentrations in the intestine for prolonged periods.

We have not been able to confirm the amoebicidal activity observed by Nakamura & Jonsson (1957) in unsubstituted dichloroacetamide; our results showed this compound to be completely inactive both *in vitro* and *in vivo*.

The antitubercular activity of the benzothiazole derivatives is not uniformly increased by dichloroacetylation of the amines.

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RELEASE OF OXYTOCIN IN UNANAESTHETIZED LACTATING RATS

BY

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The action of certain substances on the release of oxytocin has been studied in unanaesthetized lactating rats. The release of oxytocin has been assessed by the increase in weight after suckling of rats separated overnight from the mother. A large variety of substances appear to block the release of oxytocin. These include pentolinium, atropine and chlorpromazine. Adrenaline and noradrenaline also cause a block in the release of oxytocin, and it is suggested that adrenaline blocks the central release of oxytocin as well as blocking its peripheral action. Reserpine and oestradiol in the doses administered did not block the release of oxytocin. Block also occurred after the ingestion of water, ethanol and normal saline, an effect which may be produced indirectly by impulses from the gastro-intestinal tract.

It has been observed that any stimulus to the neurohypophysis releases both oxytocin and vasopressin. This has been shown in rabbits after electrical stimulation of the supraopticohypophysial tract (Harris, 1955), in dogs after injections of hypertonic saline (Abrahams & Pickford, 1954) and in rats after injections of nicotine (Walker, 1957). Very little is known about substances which block the release of the neurohypophysial hormones or whether the release of one of these hormones can be blocked independently of the other. The effect of various substances on the release of oxytocin in unanaesthetized lactating rats after suckling has been studied. The substances investigated were water and ethanol, which are known to inhibit the release of vasopressin ; the tranquillizers reserpine and chlorpromazine, which are thought to act at the level of the hypothalamus ; and oestrogen in doses which block the release of the anterior pituitary hormones. The interesting suggestion has been made by Pickford (1947) that the hypothalamic nuclei may be analogous to a peripheral autonomic ganglia in that they possess a synapse at which acetylcholine may be the chemical transmitter of afferent nerve impulses while the supraopticohypophysial tract may constitute the efferent path for the release of the neurohypophysial hormones (Walker, 1957). The action of a ganglion-blocking drug, pentolinium, and an anticholinergic drug, atropine, on the release of oxytocin has been studied by administering these substances to the lactating mother just before suckling. The mechanism of action of adrenaline and noradrenaline in blocking the milk-ejecting reflex has also been investigated.

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METHODS

Lactating rats (200 to 250 g in weight) with a litter of 4 to 5 pups were used for studying the release of oxytocin 10 to 12 days after birth of the litter. The release of oxytocin was assessed by the increase in weight after suckling of the young rats separated overnight from the mother. The pups were removed from the mother overnight and weighed next morning before suckling. They were returned to the mother for 15 min at the end of which period they were weighed again. When the weight gain of the litter was constant on several mornings the substance to be tested was administered to the mother just before suckling. If there was no increase in the weight of the litter after 25 min of feeding, then oxytocin was administered intraperitoneally to the mother in doses varying from 10 to 100 m-u. and the litter placed with the mother again. If at the end of this second period of feeding the litter increased in weight as on the other days, it was concluded that the substance administered had specifically blocked the release of oxytocin. The block has been classified as complete block, partial block or no block in the release of oxytocin. Litters were sometimes used for a second experiment, after an interval of 5 days and when the rate of increase of weight was again constant.

The drugs used were: adrenaline hydrochloride, noradrenaline hydrochloride, chlorpromazine hydrochloride (Largactil), reserpine (Serpasil), oestradiol dipropionate, atropine sulphate, pentolinium (Ansolysen), oxytocin (Syntocinon). All these drugs were administered to the mother intraperitoneally. Water and ethanol and saline were administered by mouth by means of a soft rubber catheter 15 to 35 min before suckling.

RESULTS

Water. Tap water (3.5% to 7% of body weight) when administered to the lactating mothers completely blocked milk ejection in 10 out of 12 experiments. When oxytocin in doses of 25 to 100 m-u. was administered intraperitoneally to the mother just after the first suckling the litter gained their normal increase in weight after suckling in 8 experiments and had a partial increase in weight in 2 experiments. Table 1 shows the results of 4 typical experiments when the release of oxytocin was completely blocked by the administered water, the block being overcome by subsequent administration of oxytocin showing that exogenous oxytocin was still effective.

TABLE 1

ACTION OF WATER ON THE MILK-EJECTION REFLEX

Litter	Weight of pups before feeding (in g)	% body weight of water administered to mother orally	Weight of pups after 15 min suckling	Oxytocin injected in m-u. to mother	Weight of pups after suckling again for 15 min
T	96·0	4·4%	96·0	50	99
S	96·5	4·2%	96·5	25	99·5
V	75·0	3·5%	75·0	25	78·5
C	134·5	5·5%	134·0	100	136·5

Ethanol. When 5 ml./100 g of body weight of 7.5% to 10% (v/v) ethanol was administered to the mother 15 to 30 min before feeding in 8 experiments there was no increase in the weight gain of the litter in 6 experiments and partial increase in weight in 2 experiments. Oxytocin administered in doses of 50 to 100 m-u. allowed the litter to gain their normal increase in weight after feeding in 5 experiments and allowed a partial increase in weight in one experiment. Fig. 1 depicts the results of 2 experiments indicating a block in the release of oxytocin in 2 rats

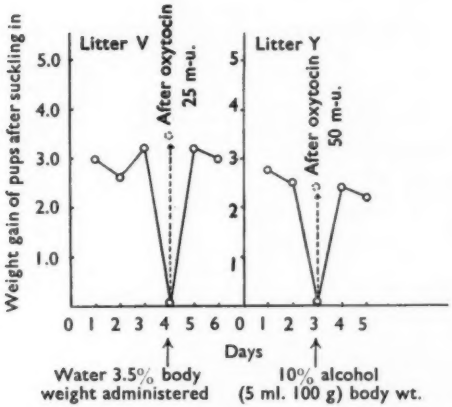

Fig. 1. Action of water and alcohol on the milk-ejection reflex.

treated with water or ethanol. The block was overcome in both cases by an intraperitoneal injection of oxytocin.

Saline. When 5 ml./100 g 0.9% sodium chloride solution was given by stomach tube, there was a block of the reflex similar to that caused by water and ethanol. When the litter was weighed after suckling at 30 min intervals, it was found that the block lasted for about 1.5 hr, after which time the pups were able to obtain about 70% of the normal amount of milk.

Adrenaline. Adrenaline when administered intraperitoneally to the lactating mother in doses of 1.0 μg and above caused a complete block in the milk-ejection reflex in 8 out of 10 experiments and a partial block in 2 experiments. The results are shown in Table 2. Subsequent administration of oxytocin (50 m-u.) overcame the block completely in 3 experiments and caused up to 70% reversal of the block in the remaining 5 experiments. In 5 experiments oxytocin (50 m-u.) was administered to the mother at the same time as adrenaline but at a different site. In 4 of these experiments milk ejection occurred (Table 2). Oxytocin when administered in doses of 10 m-u. at the same time as the adrenaline injection did not prevent the block caused by adrenaline. In 7 experiments adrenaline (1 μg) was administered 30 min after an injection of phenoxybenzamine (50 μg/kg). Adrenaline caused a complete block in the release of oxytocin in 5 of these experiments, partial block in one and no block in another experiment. In another 5 experiments acetylcholine administered in doses of 5.0 μg 2 min before the administration of 1.0 μg did not prevent the block caused by adrenaline (Table 2). When adrenaline was administered in doses lower than 0.5 μg it did not always block the lactating reflex.

Noradrenaline. In 5 experiments noradrenaline in doses of 1.0 μg administered to the lactating mother just before feeding completely blocked milk ejection in 4 experiments and caused a partial block in one experiment. After the administration of 50 m-u. oxytocin the litter gained their normal weight after suckling in 2 experiments and gained partial weight in 3 experiments (70%, 66%, 50%). The results are summarized in Table 2.

TABLE 2
ACTION OF DRUGS ON MILK-EJECTION REFLEX IN RATS

Substances administered	No. of experiments in which block occurred		
	Total block	Partial block	No block
Adrenaline 1 μg and above	8	2	0
Adrenaline 1 μg+oxytocin 50 m-u.	0	1	4
Adrenaline 1 μg after phenoxybenzamine 50 μg/kg	5	1	1
Adrenaline 1 μg after acetylcholine 5 μg	5	0	0
Noradrenaline 1 μg	4	1	0
Chlorpromazine 5 mg/kg	4	2	0
Reserpine 2·5–4·0 mg/kg	0	0	5
Pentolinium 0·1 μg	6	2	0
Pentolinium 10–50 μg+oxytocin 50 m-u.	0	0	4
Pentolinium 1 μg after acetylcholine 10 μg	0	5	4
Atropine 1 μg and above	9	1	0
Atropine 1 μg after acetylcholine 2–5 μg	7	3	1
Atropine 1 μg after acetylcholine 10 μg	2	5	3
Atropine 1 μg+oxytocin 50 m-u.	0	1	4
Oestradiol 200 μg	0	0	5

Chlorpromazine. Chlorpromazine was administered in doses of 5.0 mg/kg to 6 lactating mothers before feeding. It caused a complete block in milk ejection in 4 experiments and a partial block in 2 experiments. The block was completely overcome by subsequent administration of oxytocin to the mother in 5 out of 6 experiments. Chlorpromazine when administered in a dose of 1, 2 and 3 mg/kg did not always block the reflex.

Reserpine. Reserpine when administered to the lactating mother 30 to 60 min before feeding in doses varying from 2.5 mg/kg to 4.0 mg/kg in 5 experiments caused no block of milk ejection. Reserpine could not be administered in higher doses as it was toxic to the animals.

Pentolinium. Pentolinium when administered to the lactating mother in doses of 1.0 μg and above just before feeding always caused a complete block of milk ejection. In the experiments in which pentolinium was administered in doses of 0.1 μg it caused a complete block of milk ejection in 6 experiments and a partial block in 2 experiments. When oxytocin in doses of 50 m-u. was administered at the same time as pentolinium (10 to 50 μg) the block caused by pentolinium did not occur in any of the 4 experiments (Table 2). In 9 experiments acetylcholine (10 μg) was administered to the mother 2 min before administration of 1.0 μg of pentolinium. The acetylcholine completely prevented the block in 4 experiments and partially prevented it in 5 experiments. Fig. 2 demonstrates 2 experiments on the same litter showing pentolinium (1.0 μg) causing a complete block in the weight gain of the litter on one day and only partial block in the weight gain on the litter on another day, when acetylcholine (10.0 μg) had been administered to the mother 2 min before the administration of the same dose of pentolinium. Acetylcholine when administered in doses less than 10.0 μg did not prevent the block caused by pentolinium.

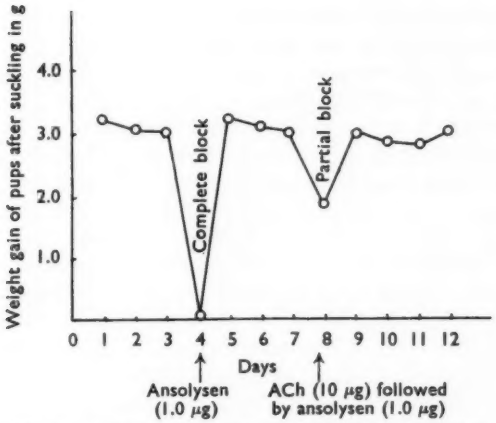

Fig. 2. Action of ansolysen on the milk-ejection reflex.

Atropine. Atropine in doses of 1.0 μg and above was administered to the lactating mother before feeding in 10 experiments. In 9 experiments milk ejection was completely blocked while in the other a partial block occurred (Table 2). Oxytocin in doses of 50 m-u. allowed the litter to gain this normal weight after suckling in all experiments. Oxytocin (50 m-u.) when administered at the same time as atropine completely prevented the block caused by atropine in 4 out of 5 experiments. Acetylcholine was administered to the mother in doses of 2 to 5 μg and 10 μg just prior to administration of atropine in 11 experiments and 10 experiments respectively. The results can be seen in Table 2.

Oestradiol. The intraperitoneal injection of oestradiol 1.5 to 3 hr before suckling failed to affect the reflex in 5 experiments (Table 2).

DISCUSSION

The simple method of measuring the release of oxytocin which has been followed in this investigation has the advantage that a physiological release of oxytocin is being measured in unanaesthetized animals. The action of ethanol and water in blocking the release of oxytocin in unanaesthetized lactating rats confirms earlier results in another species which demonstrated that the administration of water completely blocked the lactating reflex in unanaesthetized lactating rabbits (Chaudhury, 1960). Peeters, Stormorken & Vanschoubroek (1960) have recently shown that in lactating cows the effect of certain stimuli which normally caused milk ejection was inhibited by "conditions of hydration." These authors stress, however, that "conditions of hydration" indicated a complex of experimental details and procedures including the presence of a bladder catheter which may have constituted the inhibiting factor rather than hydration *per se*. The results presented above in this paper indicate that the administration of water just before suckling blocks the milk-ejection reflex. Cross (1951) in rabbits and Pickford (1960) in female dogs found that a water load did not inhibit the lactating reflex. It is possible that the time interval between hydration and the suckling may be responsible for the

apparently conflicting results. Cross had a time interval of 90 min and Pickford an interval of 2 hr between the first hydrating dose of water and the initiation of suckling. In the experiments described above only one water load was given and milk ejection assessed 15 to 30 min after the administration of water. Since it was possible that filling up of the stomach was responsible for the discrepant results, the action of normal saline was investigated. It was found that saline also blocked the reflex, and it is therefore likely that the block caused by substances given by mouth was, at least in part, due to distension of the stomach. It is interesting to note that substances which are known to inhibit the release of vasopressin, like water and alcohol, also block the release of oxytocin. It has previously been shown that both oxytocin and vasopressin are always released simultaneously in response to any stimuli, and these results indicate that a differential blocking of one of these hormones has not been possible. Dicker (1958) has shown that in rats methylpentynol, which belongs to a group of alcohols, also inhibits the release of the neurohypophysial hormones following osmotic stimulation. In women in the first stage of labour or two days after puerperium Dicker (1959) has recently demonstrated that contractions of the uterus were very little affected by ingestion of 1 l. of water or 20 to 30 ml. ethanol in 200 ml. water. It is, however, possible that the amount of water ingested was not adequate to suppress the release of oxytocin. It may also be stressed that uterine contractions are not a very reliable index of the release of oxytocin. Theobald (1959) has, however, got similar results using the suckling reflex as an index of oxytocin release. He has mentioned the importance of the time interval between hydration and the onset of suckling when antidiuresis is being studied in suckling.

The action of adrenaline inhibiting the milk-ejection reflex has been worked out in detail on lactating rabbits by Cross (1953, 1954). The present view appears to be that while there is evidence that adrenaline can block the peripheral effect of oxytocin on the mammary gland there is no evidence that adrenaline blocks the central release of oxytocin (Cross, 1957). The results on suckling rats presented in this paper indicate that adrenaline in addition to blocking the peripheral effects of oxytocin on the mammary gland also prevents the endogenous release of oxytocin in response to the stimulus of suckling.

Chlorpromazine appears to block the release of oxytocin while reserpine in the doses administered did not prevent the release of oxytocin. These results with reserpine do not agree with the findings of Moon & Turner (1959), who have shown that reserpine blocks milk ejection in lactating rats. Gaunt, Renzi, Antonchak, Miller & Gilman (1954) have also shown that reserpine does not inhibit the release of the neurohypophysial antidiuretic hormone in rats. Oestrogen, when administered to the lactating mothers in doses of 200 μg 1.5 to 3 hr prior to suckling, did not block the milk-ejection reflex in any of the 5 experiments.

The fact that the ganglion blocking drug pentolinium in as low doses as 0.1 μg completely blocked milk ejection is very interesting in view of the hypothesis (Pickford, 1947) that the hypothalamic nuclei may be analogous to a peripheral autonomic ganglion in that they possess a synapse at which acetylcholine may be the chemical transmitter. It is also intriguing to note that when acetylcholine was

administered to the lactating rat in doses of 10 μg, pentolinium 1 μg did not completely block milk ejection in any experiment. Pentolinium administered in doses of 1.0 μg to a rat without prior injection of acetylcholine caused a complete block of milk ejection in all experiments. Acetylcholine when injected in doses lower than 10 μg did not, however, prevent the block of milk ejection caused by pentolinium, and it is possible that acetylcholine when administered in doses of 10 μg may have had a milk-ejecting action of its own or caused endogenous release of oxytocin itself.

Bisset & Walker (1957), studying the action of the ganglion blocking drug, hexamethonium, on the release of oxytocin and vasopressin, found that it did not block the release of these hormones after stimulation to the neurohypophysis by nicotine. However, as the authors point out, hexamethonium itself had an antidiuretic effect, and Walker (1957) has suggested the possibility that hexamethonium may have stimulated the neurohypophysis indirectly by this fall in blood pressure. It should also be kept in mind that the stimulus in one instance was pharmacological and in the other physiological, and the responses may not be the same for these two different types of stimuli to the neurohypophysis. Much more work needs to be done investigating the action of ganglion blocking drugs on the release of the neurohypophysial hormones. The dose of pentolinium which blocked the response is very small, and it is doubtful if in doses of 0.1 μg pentolinium would block the autonomic effects in a rat of 200 to 250 g.

The results presented indicating that atropine blocks the milk-ejecting reflex confirm the earlier work by Grosvenor & Turner (1957). Acetylcholine in doses of 10 μg administered just prior to the administration of atropine prevented a complete block of milk ejection in 8 out of 10 experiments. Pickford has found that acetylcholine injected into the supraoptic nucleus produced antidiuresis in the presence of atropine. The results presented above indicate that atropine blocks the milk-ejection reflex, but it is not suggested that atropine acts by virtue of its anticholinergic action. Further work has to be done elucidating how atropine blocks milk ejection. Acetylcholine in doses of 10 μg administered just prior to the administration of atropine prevented a complete block of milk ejection in 8 out of 10 experiments. This could have been due to a direct action of acetylcholine on the myoepithelium or due to acetylcholine itself releasing oxytocin.

It is interesting that such a variety of pharmacological agents block the lactating reflex. That this block was not due merely to the injection is shown by the findings that the milk-ejection reflex was not blocked when adrenaline and noradrenaline were administered intraperitoneally at doses below 0.1 μg and chlorpromazine at doses below 0.5 mg/kg. As the doses administered were raised partial block in milk ejection occurred such as when adrenaline was administered at doses of 0.1 to 0.4 μg or when chlorpromazine was administered at 1, 2 and 3 mg/kg. When the doses were further raised there was a complete block in milk ejection in nearly all experiments. The stress of the injection therefore did not block milk ejection as otherwise it would have blocked the milk ejection in those experiments also where low doses of the drugs were administered.

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STRESS-INDUCED BLOCK OF MILK EJECTION

BY

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A simple method has been described by which the action of drugs on the stress-induced block in milk ejection can be investigated on lactating guinea-pigs. Reserpine, meprobamate and chlorpromazine when administered to the lactating mother at various periods before suckling reduced the block in milk ejection caused by the stress. Dibenamine and dichloroisoprenaline did not affect in any way the stress-induced block. It is suggested that the stress-induced block in milk ejection is probably a nervous block and not mediated through adrenaline.

It has been known for a long time that fright or nervousness inhibits proper feeding of infants and milking in lactating animals. Ely & Peterson (1941) demonstrated marked inhibition of milk ejection in frightened cows, and Newton & Newton (1948) described inhibition of milk ejection caused by fear, embarrassment and anger in a lactating subject. It has been demonstrated that intravenous administration of adrenaline or stimulation of the sympathetic areas of the hypothalamus blocks the milk-ejection reflex (Cross, 1953, 1954). Cross (1955) has produced the stress-induced block of milk ejection in lactating rabbits and studied the mechanism of this block. The present concept appears to be that the adrenaline released in stress may block milk ejection by its vasoconstrictor action on the mammary myoepithelium. This peripheral action of adrenaline has been well demonstrated (Cross, 1953). It is, however, possible that the adrenaline released in stress may prevent the release of oxytocin and also that there may be other factors concerned in the psychological block of milk ejection. The effect of drugs on the stress-induced block of milk ejection does not appear to have been investigated in experimental animals. In this investigation the effect of some of the tranquillizers and two adrenergic nerve blocking drugs on the milk-ejection reflex in stress has been studied on the lactating guinea-pig.

METHODS

Guinea-pigs were bred in the animal house and the litter of each guinea-pig reduced to two for purposes of the experiment. Experiments were carried out from the third to the tenth day after birth of the litter. The two young guinea-pigs were separated overnight from the mother and weighed next morning before suckling. They were placed with the mother for 5 min, at the end of which period they were weighed again. When the weight gain of the

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litter was constant on several mornings the mother was turned and tied on its back on the day of the experiment and the two pups allowed to feed for 5 min. They were weighed at the end of the 5 min and then allowed to feed for a further 5 min in the cage in normal surroundings. After 5 min suckling without any stress the lactating guinea-pig was given an injection of 50 m-u. oxytocin and the two pups allowed to feed for a further 5 min. It was observed in the experiments that the weight of the litter after 5 min of uninhibited suckling was not further increased by allowing them to feed again after the injection of oxytocin into the mother. This indicated that there was full milk ejection when the litter were allowed a 5-min feeding in the cage after 5 min of "stress" feeding. The block in the milk ejection has been expressed as a percentage block where the total weight gain of the litter after both "stress" and "normal" feedings on any day has been taken as 100% milk yield. It was observed that this 100% milk yield was similar in amount to the milk yield on the earlier control days. Drugs were administered to the lactating mother at various intervals before the stress-induced block in milk ejection, and the % inhibition of the stress-induced block after administration of the drugs was determined. The lactating guinea-pigs were not used for administration of a second drug until there were three to four days' control readings of milk yield after the administration of the first drug.

To ensure that there would be adequate milk in the mammary glands of the guinea-pig prolactin 5 u. was injected into the mother on alternate days in the evening. It had previously been determined that prolactin, even in large doses, did not have any action on the milk-ejection reflex.

The drugs used were: reserpine, meprobamate (Wallace Laboratories), chlorpromazine hydrochloride (Schering Research Division), dibenamine (Merck), dichloroisoprenaline, and oxytocin. In another series of experiments the effect of the following drugs on normal milk ejection in the guinea-pig was investigated: antidiuretic hormone, adrenocorticotrophic hormone, prolactin, and adrenaline hydrochloride. All the above-mentioned drugs except meprobamate were administered intraperitoneally to the lactating mother. Meprobamate was administered orally to the mother as a suspension in 0.5% methylcellulose.

RESULTS

Stress caused a block in milk ejection in all 10 experiments when the 10 lactating guinea-pigs were allowed to nurse their litter while being tied down on their backs. The % block caused by stress varied from 33% to 100%. A further 5-min suckling in the cage immediately afterwards indicated that the milk-ejection reflex had been restored. The litter gained their usual increase in weight. Fig. 1 shows the daily increase in weight of two litters. The weight increase when the mother nursed for 5 min under conditions of stress has been shown. There was a block in milk ejection in both experiments and this block disappeared when the litter were allowed to feed in the cage. In four experiments the lactating guinea-pigs received an injection of 50 m-u. oxytocin intraperitoneally immediately prior to being tied down on their back and nursing the litter. The litter gained their normal increase in weight in these experiments and there was no block in the milk-ejection reflex. This indicated that the block in milk ejection caused by tying the mother down on her back was not caused by mechanical or postural factors but was in fact due to a lack of effective circulating oxytocin. Table 1 shows how different drugs affect the block in milk ejection caused by stress.

Adrenaline, when administered to the lactating mother in a dose of 5 to 10 μg, always caused a block in the milk-ejection reflex and this block was not prevented by prior administration of dibenamine to the mother.

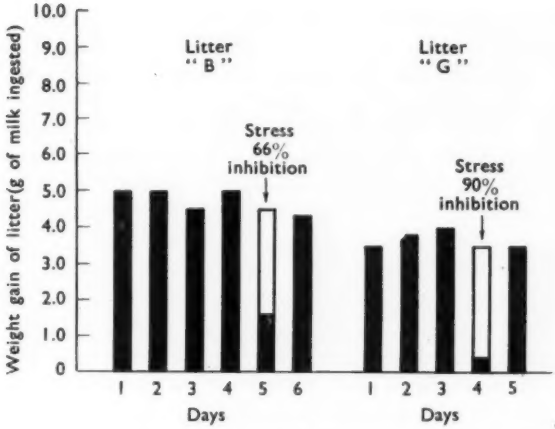

Fig. 1. The action of stress on the milk-ejection reflex in two lactating guinea-pigs. Stress was produced on the fifth and fourth day respectively. Shaded columns indicate the weight gain after 5 min suckling. Blank columns indicate the weight gain of the litter when they were allowed to feed for 5 min in normal surroundings following the stress.

Experiments were performed where adrenocorticotrophic hormone (20 u.) or antidiuretic hormone (10 m-u.) was administered to the lactating mother just before normal suckling. These drugs, which are released in stress, did not cause a block in milk ejection.

TABLE 1

PERCENTAGE BLOCK IN MILK EJECTION IN LACTATING GUINEA-PIGS

Stress	Stress after reserpine 250 μg/kg intraperitoneally 18 hr prior to suckling	Stress after meprobamate 200 mg/kg orally 30–40 min prior to suckling	Stress after chlorpromazine 2·5 mg/kg intraperitoneally immediately prior to suckling	Stress after dibenamine 200 mg/kg intraperitoneally 2 hr before suckling	Stress after dichloro-isoprenaline 10 mg/kg immediately prior to suckling
60·0	50·0	0	20·0	66·0	100·0
100·0	33·0	10·0	40·0	60·0	66·0
33·0	37·0	20·0	33·0	75·0	100·0
100·0	75·0	0	60·0	66·5	45·0
91·0	37·5	15·0	66·0	100·0	33·0
66·0	60·0	33·0	30·0	50·0	48·0
60·0	33·0				
75·0	35·0				
100·0	40·0				
75·0	37·0				

DISCUSSION

One of the purposes of this communication is to describe a simple method whereby the action of drugs on the stress-induced block of milk ejection can be studied. The guinea-pig responded to stress in a similar way every time and the milk yield after 5 min suckling was also relatively constant on different days in these litters. The results with the tranquillizing drugs indicate that reserpine, chlorpromazine and meprobamate all reduced the stress-induced block of milk ejection. In two experi-

ments meprobamate prevented the block. Reserpine could not be administered at a dose higher than 300 μg/kg because it was toxic to the guinea-pig, while chlorpromazine at a dose higher than 3.0 mg/kg itself blocked the normal milk-ejection reflex as in the rat (Chaudhury, 1961). Cross has also observed that chlorpromazine abolishes the stress-induced block of milk ejection in the rabbit (personal communication). It had been hoped that perhaps some of the tranquillizers would prevent the block in the reflex and others would not, and that this might give some indication of the site of action of this blocking phenomenon. However, this did not happen. As the investigation was not meant to be a study of the comparative merits of the tranquillizers no statistical evaluation of the three tranquillizers has been performed.

Dibenamine, when administered in doses which block the pressor response to adrenaline, did not prevent the stress-induced block of milk ejection. It could not be administered at a dose higher than 200 μg/kg, as that altered the normal milk-ejection reflex. Dichloroisoprenaline, which has been shown to block the inhibitory actions of adrenaline (Powell & Slater, 1958), also did not prevent the stress-induced block of milk ejection when administered in doses up to 10 mg/kg before suckling. These results give no further support for the hypothesis that the stress-induced block in milk ejection is mediated through the release of adrenaline from the adrenals. Although adrenaline when administered to lactating animals causes a block in the milk-ejection reflex, it is possible that other mechanisms may be involved in the immediate emotional block of milk ejection. It has been observed that the ringing of the telephone or a knock on the door during suckling causes a momentary block of milk ejection. It is doubtful if adrenaline is released under these circumstances. It may well be purely a nervous inhibition mediated through a chemical transmitter.

Two other substances thought to be released in stress, adrenocorticotrophic hormone and antidiuretic hormone, did not block normal milk ejection in the lactating guinea-pig. They could not, therefore, be concerned in the stress-induced block of milk ejection. It would be interesting to investigate whether oxytocin is released in stress. Usually it is released whenever antidiuretic hormone is released and in larger quantities (Harris, 1955). If oxytocin is not released in stress, then this would be one of the few situations where oxytocin is not released but antidiuretic hormone is (Rydin & Verney, 1938). On the other hand, if oxytocin is released in stress it becomes difficult to explain the stress-induced block in milk ejection. Cowie & Folley (1957) have recently stated that the physiological and psychological factors which may inhibit the normal milk-ejection reflex are of great scientific and practical interest. It is hoped that further investigations will elucidate the mechanism by which the stress-induced block in milk ejection occurs.

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RELATIVE ACTIVITY OF SOME INHIBITORS OF MONO-AMINE OXIDASE IN POTENTIATING THE ACTION OF TRYPTAMINE IN VITRO AND IN VIVO

BY

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Several known inhibitors of mono-amine oxidase (iproniazid, isocarboxazid, nialamide, phenelzine, pheniprazine and tranylcypromine) were tested for their ability to (i) inhibit the mono-amine oxidase activity of a rat brain mitochondrial preparation *in vitro*; (ii) potentiate the action of tryptamine on the isolated rat fundal strip preparation; and (iii) potentiate the acute toxicity of tryptamine in mice. There was some correlation between the order of potency of the drugs in the three tests, particularly in inhibiting the enzyme activity in the Warburg and in the tryptamine toxicity test in mice. Exceptions to this were isocarboxazid which had unexpectedly high activity on the rat fundal strip preparation, and tranylcypromine which was devoid of tryptamine-potentiation action on the rat fundus preparation although it inhibited rat brain mono-amine oxidase *in vitro* and potentiated the action of tryptamine *in vivo*. Tranylcypromine was considerably less active in inhibiting the mono-amine oxidase of rat fundus than rat brain tissue *in vitro*, while iproniazid and isocarboxazid had about the same potency on the enzyme from the two tissues.

When investigating the action of various analogues of tryptamine on the isolated rat fundal strip preparation, Vane (1959) observed that the addition to the organ bath of the mono-amine oxidase inhibitor β-phenylisopropylhydrazine caused a potentiation of the action of tryptamine while having no effect on the contractions due to 5-hydroxytryptamine. This was explained as being due to the penetration of the tryptamine but not 5-hydroxytryptamine inside the cell where it could be inactivated by mono-amine oxidase.

In view of the current interest in drugs that effect the metabolism of catechol and indole amines, and the possible use of these agents in the treatment of psychiatric disorders, we have attempted to develop simple pharmacological procedures for evaluating the mono-amine oxidase inhibitory activity of new drugs by assessing their ability to potentiate some actions of tryptamine.

Several known inhibitors of mono-amine oxidase were compared for their ability to (i) inhibit the mono-amine oxidase activity of a rat brain mitochondrial preparation *in vitro*; (ii) potentiate the action of tryptamine on the isolated rat fundal strip preparation; and (iii) potentiate the acute toxicity of tryptamine in mice.

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METHODS

Preparation of mono-amine oxidase from rat brain. A method similar to that described by Pletscher & Gey (1958) and Davison (1957) was used. The enzyme was prepared from the brains of freshly killed rats. The mixture of cell nuclei, mitochondria and microsomes obtained by differential centrifugation of the brain homogenates was used as the source of enzyme.

Preparation of mono-amine oxidase from rat fundus. It was difficult to obtain a satisfactory enzyme preparation from this tissue by grinding or homogenization, and therefore the following procedure was used. The fundi from a number of rats were cut into pieces and treated with a 10% solution of cetrimide for about 5 min. The tissue was then thoroughly washed in distilled water. About 0.2 g of fundal tissue treated in this manner was used in micro-flasks. In some experiments brain tissue was treated in a similar manner as a comparison.

Determination of enzyme inhibition. Tyramine or tryptamine (9×10^{-3} M) was used as substrate, and the rate of oxygen uptake at 37° C in a Warburg apparatus determined as a measure of enzyme activity. Test drugs were incubated with the enzyme preparation under standard conditions for 30 min before the addition of substrate. A thermobarometer and flasks containing enzyme preparation, substrate but no inhibitor were used as controls in all experiments. Flasks containing enzyme preparation, inhibitor but no substrate were included when required. In the experiments using micro-flasks the rate of oxygen uptake by tissue preparation plus inhibitor was determined for 20 min before tipping in the substrate. The effect of various concentrations of the test drug on the activity of the enzyme preparation was determined. From a plot of the percentage inhibition against log concentration, the concentration of test drug required, under these conditions, to reduce the activity of the enzyme preparation by 50% (I 50 value) was determined.

Isolated rat fundal strip preparation. In vitro experiments in the rat fundal strip preparation were carried out using the method described by Vane (1957). An isotonic lever of approximately 14:1 magnification was used. Drugs were added to the organ bath (15 ml.) in a volume of approximately 0.2 ml. The tryptamine was allowed to act for 1.5 min, the bath washed out, and 30 sec later washed out again. The tissue was then allowed to rest for 3 min. The interval between successive doses of tryptamine was thus 5 min. Inhibitors of mono-amine oxidase and other test drugs were added to the bath immediately after the second washing. The heights of the contractions produced by a series of concentrations of tryptamine were determined before and at intervals following the addition to the bath of various concentrations of the mono-amine oxidase inhibitor. The potentiating action of the drugs increased with the length of time they were in the organ bath. For comparative purposes, the potentiation observed after the drug had been in the bath for 45 min was determined, as this was found to be the time requirement for maximal effect with most drugs. Dose-response curves for the action of tryptamine before and after the addition of the test drug were plotted. Since these were approximately parallel it was permissible to evaluate the dose-ratios, that, is the ratio of the concentrations of tryptamine required to produce equal effects before and after addition of the test drug to the organ bath. The log of the dose-ratio was then plotted against the log of the concentration of the test drug.

In other experiments rats were injected subcutaneously with a test drug and killed 4 to 5 hr later. Fundal strips were prepared and set up in the usual manner. The heights of the recorded contractions due to various doses of tryptamine were compared with those from untreated animals.

Potentiation of tryptamine toxicity in mice. The acute subcutaneous toxicity of tryptamine was found to increase considerably if the mice were pretreated with an inhibitor of mono-amine oxidase. Groups of 10 mice were given subcutaneously various doses of the test compounds, and 18 hr later were injected subcutaneously with tryptamine at a dose (250 mg/kg) which caused no deaths in unpretreated controls. Mortalities were recorded 18 hr after the tryptamine injections and the LD50's determined from a plot of probit mortality against log dose. These experiments were carried out at an environmental temperature of 26 to 28° C. In the experiments with tranylcypromine, the tryptamine was administered 2 hr instead of 18 hr after the inhibitor.

Drugs. The following known inhibitors of mono-amine oxidase were used: Iproniazid phosphate (Roche Products), isocarboxazide (Roche Products), nialamide (Pfizer), phenelzine dihydrogen sulphate (W. R. Warner & Co.), tranylcypromine sulphate (Smith, Kline & French) and pheniprazine hydrochloride.

RESULTS

Mono-amine oxidase inhibitory activity in vitro

Rat brain. Table 1 lists the concentration of drug (I 50) which was required to produce 50% inhibition of rat brain mono-amine oxidase using tyramine as substrate. Johnston (personal communication) has confirmed that with three of the inhibitors

TABLE 1

RELATIVE ACTIVITY OF VARIOUS DRUGS IN INHIBITING MONO-AMINE OXIDASE *IN VITRO* AND POTENTIATING THE ACTION OF TRYPTAMINE *IN VITRO* AND *IN VIVO*

Figures in brackets refer to the number of experiments. (i) In the manometric experiments the inhibitors were incubated for 30 min with the brain mitochondrial preparation before addition of the substrate (tyramine 9×10^{-3} M). Oxygen uptake was corrected for thermobarometer changes and uptake by the preparation without substrate. Values quoted are the mean I 50 values (concentration required to produce 50% inhibition) with the standard deviation. (ii) The concentration of drug (EC) required to produce a fourfold potentiation of the action of tryptamine on the isolated rat fundal strip preparation. (iii) The dose of compound (ED) which, when given 18 hr previously, caused 50% mortality in mice receiving 250 mg/kg of tryptamine subcutaneously. Tranylcypromine was given 2 hr before the tryptamine

	Inhibition of rat brain mono-amine oxidase *in vitro*		Tryptamine potentiation of rat fundus		Tryptamine potentiation in mice	
Drug	I 50 (μM) (i)	Potency ratio	EC (μM) (ii)	Potency ratio	ED (mg/kg) (iii)	Potency ratio
Iproniazid	16±0·1 (20)	1·0	4·7±0·3 (8)	1·0	55±10 (3)	1·0
Nialamide	7·8±0·5 (2)	1·9	0·18±0·1 (4)	26	4±0·5 (3)	14
Isocarboxazid	4·8±0·9 (4)	3·1	0·05±0·02 (8)	94	8±2 (1)	7
Phenelzine	0·9±0·1 (2)	18	0·09±0·02 (4)	55	4±1 (1)	14
Pheniprazine	0·53±0·02 (2)	31	0·13±0·04 (6)	36	1·0±0·2 (2)	55
Tranylcypromine	0·36 (1)	45	Inactive from 0·6–50 μM (6)	—	1·3±0·5 (2)	42

used in this work (phenelzine, isocarboxazid and tranylcypromine) the potency ratios are not significantly altered if tryptamine rather than tyramine is used as substrate, although the absolute I 50 values change.

Rat fundus. Cetrimide was found to be a very useful agent for obtaining an enzyme preparation from the fundal tissue. Cetrimide is known to disrupt the permeability of the membrane of some cells so that molecules of small molecular weight easily pass in or out of the cell (Hotchkiss, 1946). The rate of oxidation of tryptamine by fundal tissue treated in this manner was found to be 116±3 μl. oxygen/g tissue/hr. This figure agrees well with the figure of 124 μl. oxygen/g/hr quoted by Vane (1959) using an enzyme preparation obtained by homogenization and dialysis of the fundal tissue. With brain tissue treated with cetrimide in an identical manner the rate of oxygen uptake using tryptamine as substrate was 450± 10 μl. oxygen/g tissue/hr, which agrees reasonably with the figure of 380±60 μl. oxygen/g/hr obtained using the mitochondrial preparation. In the presence of various concentrations of three amine oxidase inhibitors these rates of oxygen

uptake were reduced (Table 2). Whereas both iproniazid and isocarboxazid inhibited both the brain and fundus preparations to an approximately equal extent, tranylcypromine was considerably more active as an inhibitor of the brain preparation. Furthermore, the slope of the percentage inhibition-tranylcypromine concentration curve was much flatter for fundal tissue than for brain tissue.

TABLE 2

RELATIVE EFFECTIVENESS OF TRANYLCYPROMINE, IPRONIAZID, AND ISOCARBOXAZID IN INHIBITING THE MONO-AMINE OXIDASE ACTIVITY OF RAT BRAIN AND FUNDAL TISSUE *IN VITRO*

Brain and fundal tissue were treated in an identical manner, with cetrimide, thoroughly washed, and incubated with phosphate buffer in manometric flasks. The inhibitors were incubated with the tissue preparations for 30 min before addition of tryptamine (9×10^{-3} M). The rate of oxygen uptake (corrected for no-substrate uptake) was 450 ± 10 µl. oxygen/g tissue/hr for the brain preparation and 116 ± 3 µl. oxygen/g tissue/hr for the fundal tissue. Figures are the mean values together with the standard deviation. * Single experiments

Drug	Molar concentration	% inhibition of tryptamine oxidation	
		Brain	Fundus
Tranylcypromine	2×10^{-7}	33 ± 12	0
	8×10^{-7}	61 ± 3	0
	3×10^{-6}	95 ± 1	26 ± 3
	3×10^{-5}	100	54 ± 4
	1×10^{-4}	—	$63+3$
Iproniazid	2×10^{-5}	26*	29 ± 1
	8×10^{-5}	63 ± 3	53 ± 2
Isocarboxazid	10^{-5}	14 ± 2	11*
	4×10^{-5}	46 ± 5	47*

Potentiation of the action of tryptamine on the isolated rat fundal strip preparation

In vitro *experiments.* Fig. 1 is part of the record of an experiment showing the increased sensitivity of the rat fundal strip preparation to the action of tryptamine caused by the addition of isocarboxazid to the organ bath. It will be noticed that this effect increased with time, and for comparative purposes the potentiation produced after the test drug had been in the bath for 45 min was used. When the log of the dose-ratio was plotted against log of the concentration of mono-amine oxidase inhibitor straight lines were obtained (Fig. 2), and these were approximately parallel for the drugs tested. This enabled potency ratios to be determined by comparing the concentrations of the test drug required to produce a fourfold increase in the sensitivity of the rat fundal strip (Table 1).

Tranylcypromine is not included in Fig. 2, because, although concentrations ranging from 0.02 to 50 µM were used, there was no reproducible increase in the sensitivity of the rat fundal strip preparation to the action of tryptamine. With the higher concentrations of the drug a transient increase in sensitivity was sometimes observed followed by a decrease in sensitivity.

Amongst the other drugs tested for their effect on the responses to tryptamine on this preparation were cocaine hydrochloride, dexamphetamine sulphate, imipramine hydrochloride and pyrogallol. Cocaine (0.13 to 1.3 µM) produced no effect at low concentrations and only a slight inhibition of the tryptamine responses at the high ones. Dexamphetamine sulphate in concentrations less than 0.25 µM

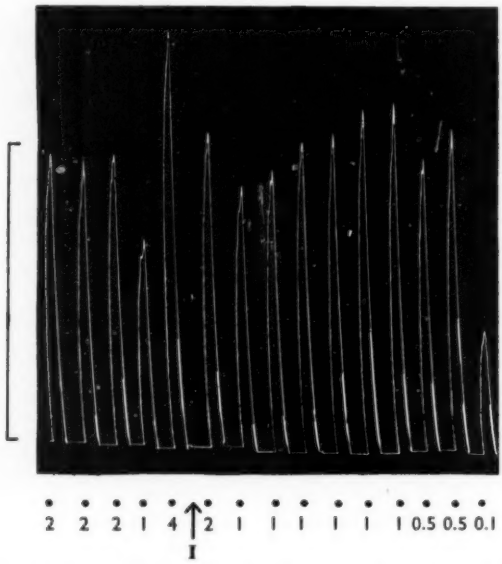

Fig. 1. Contractions of the isolated rat fundal strip preparation produced by tryptamine. The addition of isocarboxazid (0·01 μg/ml.) to the bath caused a progressive increase in the sensitivity of the preparation to tryptamine. Tryptamine added at intervals of 5 min. At black dots tryptamine in the number of μg indicated was added to the bath. At I, isocarboxazid (0·01 μg/ml.) added to bath and subsequently added after each washing out. Vertical scale, 10 cm.

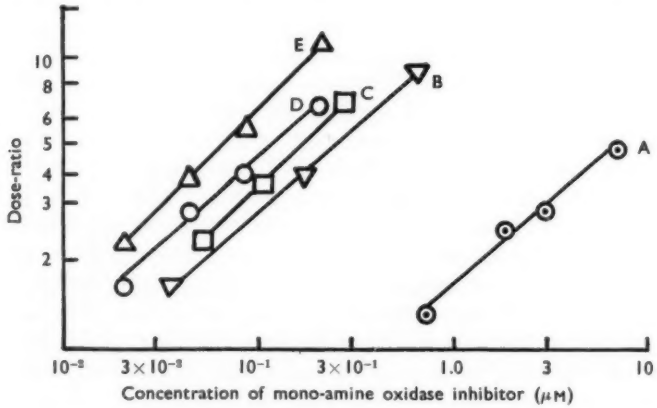

Fig. 2. Dose-response curves for the potentiation produced by various inhibitors of mono-amine oxidase of the action of tryptamine on the isolated rat fundus preparation. The dose-ratio is the ratio of the concentration of tryptamine required to produce equal responses of the preparation before and 45 min following the addition of the test drug to the organ bath. Ordinate: log dose-ratio. Abscissa: log concentration of mono-amine oxidase inhibitor (μM). A=iproniazid; B=nialamide; C=pheniprazine; D=phenelzine; E=isocarboxazid.

had no effect on the tryptamine responses. At concentrations greater than 25 μM dexamphetamine produced contractions of the fundus preparation. Imipramine hydrochloride at concentrations of 0.03 to 0.3 μM had no effect, whereas concentrations of 3 μM produced inhibition of the tryptamine responses. Pyrogallol (0.4 to 8 μM) did not modify the tryptamine responses.

In vivo/in vitro *experiments.* Fig. 3 shows the recorded heights of the contractions due to various concentrations of tryptamine of the fundal strips prepared from normal and drug-treated rats. Under these conditions tranylcypromine caused a

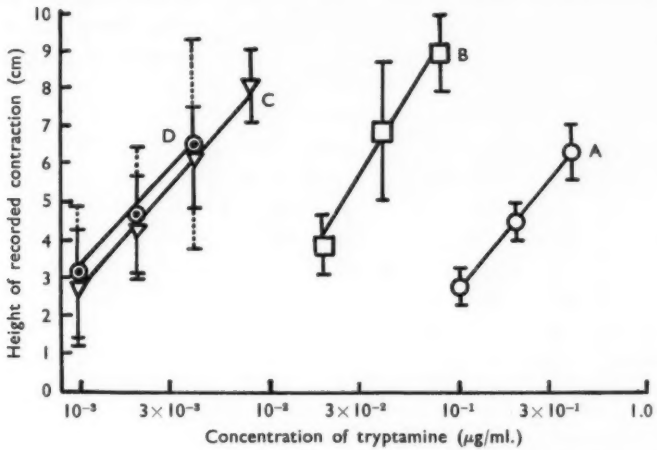

Fig. 3. Dose-response curves of the sensitivity to tryptamine of strips of fundus removed from rats injected 4 to 5 hr previously with 5.0 mg/kg subcutaneously of various mono-amine oxidase inhibitors. Ordinate: height of recorded contraction of tissue (cm; 14 : 1 magnification). Abscissa: concentration of tryptamine (μg/ml.) in organ bath (log scale). The vertical lines are the standard deviations about the mean. A=controls; B=iproniazid; C=isocarboxazid; D=tranylcypromine.

marked increase in sensitivity of the fundal strip. This increase in sensitivity was greater than that produced by the same dose of iproniazid. Since the effect of only one dose of inhibitor was studied in this manner, it is not possible to compare potencies. However, the results show that, whereas tranylcypromine does not potentiate the action of tryptamine in the isolated organ bath experiments, potentiation considerably greater than that produced by the same dose of iproniazid is obtained if the compound is injected into the rat before removal of the fundus.

Potentiation of tryptamine toxicity in mice

The acute subcutaneous LD50 of tryptamine hydrochloride in mice kept at an environmental temperature of 28 to 30° C was found to be about 500 mg/kg, death occurring without any marked symptoms of central stimulation. After subcutaneous administration of a mono-amine oxidase inhibitor, however, the LD50 was reduced, the extent of this reduction depending on the dose of inhibitor. For example, 18 hr after the administration of 1/10th the LD50 of nialamide or phenelzine, the

LD50 of tryptamine was reduced from 500 to 85 mg/kg. Furthermore, the mice showed pronounced symptoms of central stimulation, including tremor, convulsions, and marked agitation suggesting that the increased toxicity might be linked with a central action of the tryptamine. There was, however, no increase in co-ordinated motor activity such as is seen after amphetamine administration.

Fig. 4 shows plots of the percentage mortality due to 250 mg/kg tryptamine in mice 18 hr after the administration of various inhibitors of mono-amine oxidase. Although all these lines are not parallel, for comparative purposes the dose of each mono-amine oxidase inhibitor required to produce 50% mortality due to the administration of tryptamine was determined, and the results are set out in Table 1. In this test tranylcypromine substantially potentiated the action of tryptamine.

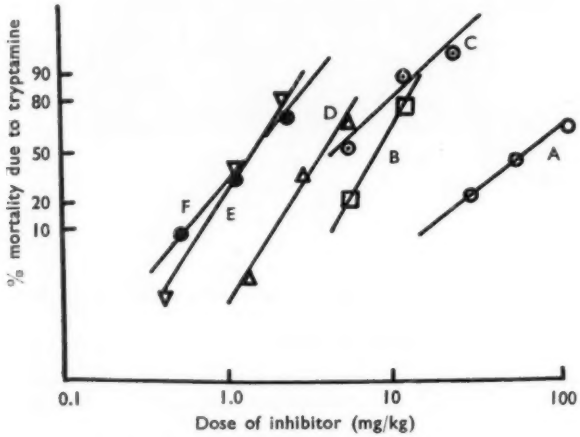

Fig. 4. Dose-response curves for the potentiation produced by various inhibitors of mono-amine oxidase of the acute toxicity of tryptamine in mice. Test drugs (with the exception of tranylcypromine) were administered subcutaneously 18 hr before tryptamine (250 mg/kg subcutaneously). Tranylcypromine was given 2 hr before the tryptamine. Ordinate: % mortality following tryptamine administration (probability scale). Abscissa: dose of mono-amine oxidase inhibitor (mg/kg). A=iproniazid; B=isocarboxazid; C=nialamide; D=phenelzine; E=pheniprazine; F=tranylcypromine.

In similar experiments the acute subcutaneous toxicity of tyramine and β-phenylethylamine was also greater in mice pretreated with inhibitors of mono-amine oxidase. The mice showed symptoms of central excitation, there was an increase in locomotor activity, vocalization and a series of escape and defence encounters similar to those seen in mice after amphetamine and described by Chance (1946). The excitement was quite different from that produced by tryptamine in mice similarly pretreated with a mono-amine oxidase inhibitor.

Comparison of the relative activities of the drugs in the three tests

The correlation between the order of activity of the compounds in the three tests was not close (Table 1) although fairly good correlation was obtained between the

inhibition of rat brain mono-amine and tryptamine potentiation in mice. Two results required explanation: (a) the complete lack of activity of tranylcypromine, and (b) the high activity of isocarboxazid, on the rat fundal strip preparation.

If the mechanism proposed by Vane (1959) for the potentiating action of mono-amine oxidase inhibitors on the rat fundal strip preparation is correct, then these differences might be attributable to one or both of two factors: (i) differences in the sensitivity to inhibitors between the mono-amine oxidase prepared from the rat fundus tissue and that from rat brain; and (ii) differences in the ability of the drugs tested to penetrate into the cell of the fundal tissue and reach the enzyme.

The difference in enzyme sensitivity mentioned in (i) might be due to (a) a difference in the nature of the mono-amine oxidase in the two tissues, or (b) metabolic alteration of some of the drugs by one tissue preparation but not by the other.

The data set out in Table 2 show that there is a marked difference in the sensitivity of rat brain and fundal tissue to tranylcypromine, whilst there is little difference in sensitivity to iproniazid and isocarboxazid.

As an indication of the ability of isocarboxazid, tranylcypromine and iproniazid to penetrate into the cell of the rat fundal tissue when added to the Tyrode solution, we measured their partition ratios between chloroform and Tyrode solution. It has been shown that the barrier which separates plasma from the gastro-intestinal tract has the characteristics of the lipoid membrane, and is preferentially permeable to the oil-soluble undissociated forms of drugs (Brodie & Hogben, 1957; Shore, Brodie & Hogben, 1957; Hogben, Tocco, Brodie & Schanker, 1959). The results obtained are set out in Table 3. Isocarboxazid had a partition ratio approximately

TABLE 3

RELATIVE POTENCIES OF VARIOUS DRUGS (IPRONIAZID=1) IN INHIBITING THE MONO-AMINE OXIDASE OF RAT FUNDUS *IN VITRO*, IN POTENTIATING THE ACTION OF TRYPTAMINE ON THE RAT FUNDAL STRIP PREPARATION, AND THEIR PARTITION COEFFICIENTS BETWEEN CHLOROFORM AND TYRODE SOLUTION (*p*H 7·4)

Drug	Inhibition of fundal mono-amine oxidase *in vitro*	Partition coefficient chloroform/ Tyrode	Tryptamine potentiation on rat fundus
Iproniazid	1	0·9	1
Isocarboxazid	1·8	100	94
Tranylcypromine	2·2	3·8	Nil

100 times that of iproniazid, suggesting that the high relative activity of the former drug in potentiating the action of tryptamine on the rat stomach strip preparation might be due to its greater ease in penetrating into cells. However, tranylcypromine also had a fairly high partition ratio (3.8), suggesting that this drug could also penetrate inside the cells of the fundal tissue.

The experiments mentioned above suggested that the lack of activity of tranylcypromine in potentiating the action of tryptamine on the isolated rat fundus preparation was related to a lack of sensitivity of the mono-amine oxidase in this tissue to inhibition by tranylcypromine *in vitro*. However, it was also possible that the potentiating action of inhibitors of mono-amine oxidase might involve some quite unspecific action such as alteration of permeability of the cells of the fundal

tissue. In order to investigate this and to attempt to establish a direct correlation between inhibition of the amine oxidase of the rat fundus in the isolated organ bath and increased sensitivity to tryptamine, the following experiments were carried out.

The degree of potentiation produced by various drugs of the action of tryptamine on the rat fundal strip in the isolated organ bath was determined in the usual manner. The strip was then removed, cut into pieces and its mono-amine oxidase activity determined in the Warburg apparatus using tryptamine as substrate. Strips of fundal tissue whose sensitivity to tryptamine had been determined but which had not been treated with an inhibitor of mono-amine oxidase were used as controls. Results of these experiments are set out in Table 4. There was a reasonably good

TABLE 4

CORRELATION BETWEEN THE INCREASED SENSITIVITY OF RAT FUNDAL STRIP TO THE ACTION OF TRYPTAMINE AND INHIBITION OF THE MONO-AMINE OXIDASE OF THIS TISSUE

The inhibitors were added to the isolated organ bath and the tryptamine potentiation determined in the usual way. The fundal tissue was then removed, treated with cetrimide, and the mono-amine oxidase activity measured in the Warburg apparatus using tryptamine as substrate. Tranylcypromine (10 μM) reduced the sensitivity of the isolated fundal strip

	Concentration in organ bath μM	Tryptamine potentiation (dose-ratio)	% inhibition of rat fundal mono-amine oxidase
Iproniazid	2·8	2·0	8
	5·6	3·5	53
	11·2	5·2	90
Isocarboxazid	0·022	2·3	44
	0·044	3·8	52
	0·09	5·9	96
Tranylcypromine	0·02	Nil	Nil
	0·08	Nil	Nil
	0·32	Nil	Nil
	2·0	Nil	Nil
	10·0	Nil	31

correlation between the inhibition of the mono-amine oxidase of the fundal tissue and tryptamine-potentiating action, suggesting that these two effects were causally related. With tranylcypromine, inhibition of mono-amine oxidase was only observed at the highest concentration tested at which a slight reduction in the sensitivity of the fundal strip to tryptamine was recorded.

DISCUSSION

The results presented in this paper suggest that testing drugs for their ability to potentiate the action of tryptamine both *in vitro* and *in vivo* may be a useful method of examining potential mono-amine oxidase inhibitors. Tedeschi, Tedeschi & Fellows (1959) have used the potentiation of the convulsant action of tryptamine in rats as a method of assaying mono-amine oxidase inhibitors, while Sjoerdsma, Oates, Zaltzman & Udenfriend (1959) suggest that the assay of urinary tryptamine may be taken as an index of mono-amine oxidase inhibition in man.

The three test systems used in this investigation were of increasing complexity. Thus in the *in vitro* experiments carried out in the Warburg apparatus, there was probably little diffusion barrier between the test drug and the enzyme system. In the isolated organ-bath experiments, the inhibitor had first to penetrate into the cell before coming into contact with the amine oxidase. In the intact animal, the situation was still more complex since there are probably two or more diffusion barriers to cross, and the inhibitor may be metabolically altered or inactivated by other enzyme systems before reaching the intracellular mono-amine oxidase.

The activities of two drugs, isocarboxazid and particularly tranylcypromine, deserve special attention. Isocarboxazid was only about twice as potent as iproniazid in inhibiting the mono-amine oxidase activity of rat fundal tissue *in vitro* using tryptamine as substrate, but was some 70 times as potent in potentiating the action of tryptamine on this tissue in the isolated organ bath. In view of the high chloroform/buffer partition ratio of isocarboxazid, the simplest explanation of this high activity is that the drug penetrates into the cell of the fundal tissue more easily than does iproniazid. The possibility that the drug may be metabolized in the tissue to a more active substance has not been ruled out.

Iproniazid and isocarboxazid were about equiactive in inhibiting the mono-amine oxidase activity of rat brain and rat fundal tissue. Tranylcypromine was therefore of particular interest, for against the fundal enzyme it had only 1/100th of its activity against the brain amine oxidase. This may be due to a difference in the mono-amine oxidase in fundal and brain tissue. The lack of activity of tranylcypromine in potentiating the effect of tryptamine on the rat fundal preparation fits in well with this idea. In the high concentrations required to produce effective inhibition of the enzyme in the organ bath the drug appears to reduce the sensitivity of the preparation by some unspecific means. Barlow (1961) has already suggested that the mono-amine oxidase in rat fundus differs from that in guinea-pig liver.

Another explanation would be that tranylcypromine is metabolized *in vivo* to a more active substance and that the enzymes required for this are present in the brain preparation but not in the fundal tissue. This is supported by the experiments where prior injection of tranylcypromine to rats greatly increased the sensitivity of the fundus to tryptamine. This increase in sensitivity was larger than that produced by the same dose of iproniazid.

The two explanations of the results are not mutually exclusive ; there may be both a difference in the enzymes from brain and fundus and also some metabolism of tranylcypromine to a more active substance.

After inhibition of mono-amine oxidase, tyramine and phenylethylamine produced a central stimulation resembling that of amphetamine, whereas the central effects produced by tryptamine were of a different type. As amphetamine contains an alpha-methyl group it is not readily inactivated by mono-amine oxidase Similarly, when the amine oxidase is inhibited, tyramine and phenylethylamine are not readily inactivated, and will therefore have a prolonged action. Thus the resemblance between the central action of amphetamine and of tyramine or phenylethylamine after mono-amine oxidase inhibition may be due to a similar central sympatho-

mimetic action of these substances. Furthermore, the difference between tryptamine after mono-amine oxidase inhibition and amphetamine suggests that amphetamine and tyramine are acting on similar "adrenergic" receptors in the central nervous system, and that these are different from the receptors acted on by tryptamine.

The fact that tranylcypromine, a known inhibitor of mono-amine oxidase, does not potentiate tryptamine on the isolated rat fundal strip preparation *in vitro* limits the value of this procedure for evaluating potential inhibitors of mono-amine oxidase, although it gives useful information with many drugs. It must, of course, be considered as a complementary test to other procedures, such as the manometric method and the tryptamine toxicity test. It is possible that the fundal strip prepared from a rat pretreated with mono-amine oxidase inhibitors, as described above, would constitute a more satisfactory routine procedure for examining new compounds. Bearing these limitations in mind, the examination of drugs for their ability to potentiate the action of tryptamine both *in vitro* and *in vivo*, as described in this paper, may be a useful method of assessing pharmacologically the mono-amine oxidase inhibitory activity of new substances.

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ANTHELMINTIC ACTIVITY OF METHYRIDINE AGAINST EXPERIMENTAL NEMATODE INFECTIONS IN MICE

BY

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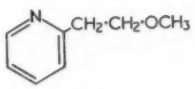
Critical studies using mice infected experimentally with nematodes (*Nematospiroides dubius, Nippostrongylus muris* and *Heterakis spumosa*) have shown methyridine to be an extremely effective anthelmintic when administered orally or subcutaneously. Comparisons of the two treatment regimes show subcutaneous administration to be the more efficient in terms of absolute dose, but neither treatment has a significant advantage in therapeutic safety margin. Methyridine has a more uniform anthelmintic action than either phenothiazine or bephenium against adult forms of the three test nematodes. In addition the drug possesses activity against all stages of certain immature nematode infections.

Although certain pyridine alkaloids possess anthelmintic properties, no simple pyridine derivative had shown useful activity until the recent paper of Broome & Greenhalgh (1961) describing the effects of 2-(β-methoxyethyl)pyridine (methyridine)



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(I) (active principle of " Promintic," Imperial Chemical Industries). It was shown that methyridine has considerable anthelmintic activity against experimental nematode infections of mice.

Methyridine may be prepared by a method similar to that used for the corresponding ethyl homologue by Doering & Weil (1947). It is a colourless, sweet-smelling liquid (b.p. = 94 to 96° C/17 mm, sp. gr.$^{20°}$ = 0.988, $n_D^{20°}$ = 1.4975) and is completely miscible with water and common solvents in all proportions. In addition the compound is a weak base (pK_a approximately 5.5) which forms a water-soluble hydrochloride (m.p. 104 to 105° C), hydrobromide (m.p. 84 to 86° C) and picrate (m.p. 130 to 131° C).

In the present paper detailed anthelmintic studies with nematode infections of mice are described, including comparative tests with other anthelmintics and some toxicological work.

METHODS

Nematodes. All tests were carried out using one or more of the three nematodes *Heterakis spumosa, Nippostrongylus muris* and *Nematospiroides dubius.* Cultures of *H. spumosa* and *N. muris* were maintained in rats by the methods of Smith (1953) and Brackett & Bliznick (1949) respectively. *N. dubius* was cultured in exactly the same way as *N. muris* except that mice, instead of rats, were used as host animals and they were infected orally.

Trials with adult nematode infections in mice. The methods used for these trials were essentially those developed earlier in these laboratories by Dr D. G. Davey. They have not previously been described and a full account of them is therefore included. To conserve animals, time and materials, each mouse received two nematode infections consisting of *H. spumosa* in the large intestine and either *N. muris* or *N. dubius* in the small intestine. Care was taken to ensure that all species reached maturity together so that none were being naturally eliminated at the time of the trial. This was achieved by infecting newly weaned mice (13 to 15 g and reared under pathogen-free conditions) with 350 embryonated *H. spumosa* eggs given orally 28 days before the experiment was due to commence. Sixteen days later half the mice were infected with 100 *N. dubius* larvae orally and seven days before the commencement of the experiment the remaining mice received 500 *N. muris* larvae together with 1.25 mg cortisone acetate (both given subcutaneously). Thus at the commencement of the experiment the infections of *H. spumosa* were 28 days old, those of *N. dubius* 12 days old and *N. muris* 7 days old, and all were at the early adult stage.

Experimental design. Three separate balanced factoral experiments involving over 800 mice were used to compare the anthelmintic activity of methyridine (oral and subcutaneous injection), bephenium (a mixture of 33% bephenium embonate + 67% bephenium hydroxynaphthoate) and fine-particle phenothiazine. The first two trials were needed to compare the dose/parasite mortality relationships of the three drugs, using doses graded from those having no activity against the parasite to those producing toxic effects in the host. The third experiment was concerned solely with providing additional information on the relative activity of methyridine when administered orally and subcutaneously.

Groups of 10 mice (all infected with *H. spumosa* but 5 containing *N. muris* and 5 *N. dubius* in addition) were randomly assigned to each dosage regime. A similar group of 10 mice served as control in each experiment to provide an estimate of the worm burden before treatment.

Administration of drugs. All drugs were administered in a single dose to provide information on the type of activity likely to be achieved under practical conditions in the field. The appropriate dose of drug was prepared in 0.5 ml. of a dispersing agent for those animals dosed orally but in 0.2 ml. for the subcutaneous treatments. The dispersing agent contained 0.1% Lissapol C, 0.1% Lissapol N.X. and 0.1% Dispersol O.G. All treatments were administered on Day 1 of the experiment.

Assessment of anthelmintic activity. Anthelmintic activity was assessed by comparing the worm burdens of treated and control animals at post-mortem examination 5 days after treatment. To facilitate worm-counting the animals were starved 24 hr prior to sacrifice. After killing, the abdomen was opened and the large and small intestines were removed to separate petri-dishes. Each large intestine was slit longitudinally, and all *H. spumosa* worms were scraped into warm water. They were then transferred to a transparent container (with transverse lines to facilitate counting) and counted over a black tile.

The small intestines containing *N. muris* were compressed between perspex plates and the nematodes were counted with the aid of an illuminated magnifying-glass over a white tile. To assess the number of *N. dubius* it was necessary to slit the upper part of the small intestine and to scrape the worms into warm water. They were then gently teased apart and counted in a transparent dish over a white tile.

Trials with immature nematode infections of mice. These trials were confined to the relatively rapid maturing nematodes *N. muris* and *N. dubius.* Equal numbers of clean mice were infected with either *N. muris* or *N. dubius* larvae, using the techniques previously

described. A single experiment involving about 250 mice was used to investigate the anthelmintic activity of graded doses of methyridine (subcutaneous injection) and bephenium (oral) against 1-, 2-, 4-, 6- and 8-day-old infections respectively. Groups of 10 mice (5 infected with *N. muris* and 5 with *N. dubius*) were randomly assigned to each treatment and 10 similar mice served as controls. All drugs were administered as previously described, and anthelmintic activity was assessed by worm counts made at post-mortem examination 12 days after infection.

Acute toxicity. The standard technique for estimating acute drug toxicity has been used in these experiments. At least 5 groups of 10 mice (of the same weight and infected with nematodes as in the anthelmintic tests) were used to assess the toxicity of graded doses of the three anthelmintic drugs.

Statistical techniques. All statistical analyses have been kindly carried out by our colleague Dr O. L. Davies, using the probit mortality/log dose relationship to assess the toxicity of these drugs to parasites and hosts.

RESULTS

The relative efficiency of methyridine, bephenium and phenothiazine against adult nematode infections in mice. The results of anthelmintic and toxicity trials are presented in Table 1 together with two indices of chemotherapeutic efficiency. These are based on the ratio between doses of drug killing 50% of the hosts and 50% of the parasites (LD50/ED50 ; Table 1a) and those killing 5% of the hosts and 95% of the parasites (LD5/ED95 ; Table 1b).

Of the drugs tested, only methyridine possessed useful anthelmintic activity when administered by subcutaneous injection. Any given dose of the drug was significantly more effective when given subcutaneously than orally (Table 1). Whether injected subcutaneously or administered orally, methyridine was a more efficient anthelmintic than either bephenium or phenothiazine in terms of absolute dose.

Since there are large differences in the relative toxicity of these three anthelmintic drugs it is necessary to compare the therapeutic ratios to evaluate their comparative merit. The data presented in Table 1 indicate that the therapeutic ratios for treating parasitized mice with methyridine are similar for all three test nematodes. By contrast, the other drugs vary widely in their effectiveness against the same test organisms. As a result methyridine is the only drug among those tested which will almost completely eliminate all test infections at a safe therapeutic dose.

The anthelmintic activity of methyridine and bephenium against immature nematode infections of mice. The results of these studies indicate that subcutaneously administered methyridine possesses marked anthelmintic activity against certain immature nematode infections of mice. Comparisons with bephenium show methyridine to be the more active drug in terms of absolute dose. Thus the ratios for doses of methyridine and bephenium needed to produce approximately the same degree of anthelmintic activity were 1/3.2 (*N. muris*) and 1/4.0 (*N. dubius*) respectively. This compares with the corresponding ratios of 1/1.3 and 1/6.3 for the adult forms of these nematodes (Table 1a).

The anthelmintic activity of the two drugs administered in doses of equal toxicity to the host (approximately 50% of LD5) is shown in Fig. 1. The results obtained from the previous experiment with 12-day-old *N. dubius* infections have been included for completeness.

TABLE 1A

THE ANTHELMINTIC ACTIVITY AND TOXICITY OF METHYRIDINE (SUBCUTANEOUS AND ORAL), BEPHENIUM AND PHENOTHIAZINE

LD50's, ED50's and their standard errors

	Toxicity	*Heterakis spumosa*		*Nippostrongylus muris*		*Nematospiroides dubius*	
Anthelmintic drug	LD50 (mg/25 g)	ED50 (mg/25 g)	Therapeutic ratio	ED50 (mg/25 g)	Therapeutic ratio	ED50 (mg/25 g)	Therapeutic ratio
Methyridine (subcutaneous injection)	41±2·5%	2·7±10%	15±10·5%	4±8·5%	9·5±9%	7±8·5%	6±9%
Methyridine (oral)	70±17%	5±10·5%	13·5±20·5%	5±9·5%	13·5±20%	9±10%	7·5±20·5%
Bephenium	197±13%	12±12·5%	16±18·5%	5·5±13%	34·5±19%	43±12·5%	4·5±18·5%
Phenothiazine	458±7%	8·5±12·5%	53·5±14·5%	21±33%	22±34%	No action	—

TABLE 1B

THE ANTHELMINTIC ACTIVITY AND TOXICITY OF METHYRIDINE (SUBCUTANEOUS AND ORAL), BEPHENIUM AND PHENOTHIAZINE

LD5's, ED95's and their standard errors

	Toxicity	*Heterakis spumosa*		*Nippostrongylus muris*		*Nematospiroides dubius*	
Anthelmintic drug	LD5 (mg/25 g)	ED95 (mg/25 g)	Therapeutic ratio	ED95 (mg/25 g)	Therapeutic ratio	ED95 (mg/25 g)	Ther? ic
Methyridine (subcutaneously)	21·5±6%	8±9·5%	2·5±11·5%	9·5±8·5%	2·5±10·5%	15±8%	±10%
Methyridine (oral)	23±24%	16±9%	1·5±26%	11·5±8%	2·0±26%	20±10%	1±26·5%
Bephenium	64·5±23%	36±13·5%	1·5±27·5%	12·5±11%	5±26%	93±12%	0·5±27%
Phenothiazine	221·5±12·5%	25±15·5%	8·5±20·5%	50·5±44%	4·5±47%	No action	—

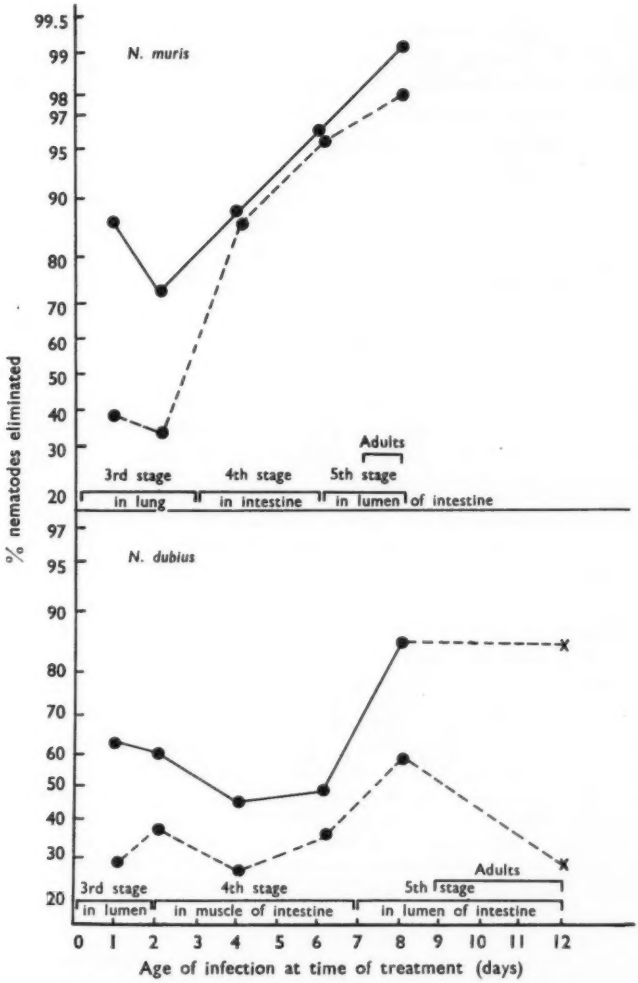

Fig. 1. Effect of methyridine subcutaneously and bephenium (oral) on immature nematode infections in mice. ●——● methyridine (10 mg/25 g). ● - - - ● bephenium (32 mg/25 g).

Fig. 1 shows that methyridine tends to give slightly better results than the equivalent dose in terms of toxicity of bephenium against immature *N. muris*, particularly when administered soon after infection. In addition, methyridine appears clearly more efficient than bephenium against immature *N. dubius* of all ages. However, these conclusions can only be regarded as indications when the standard errors attached to the toxicity data (Table 1b) are considered.

DISCUSSION

The anthelmintic studies described in this report indicate clearly that methyridine possesses considerable activity against both adult and immature nematode infections of mice. Examination of the results shows the drug is generally more active against adult worms than either phenothiazine or bephenium, two of the anthelmintics most commonly employed in veterinary practice. The margin of safety between toxic and therapeutic doses of methyridine is not so great as that for the other drugs when considered in relation to selected individual nematode species. However, considering the three test nematodes as representing a mixed natural infection, methyridine is the only compound (of those tested) which will almost completely cure mice at a safe therapeutic dose. It is this uniformity of action against the three nematode species examined, irrespective of their habitat or location in the alimentary canal, which makes the drug unique in our experience.

Another interesting feature associated with the anthelmintic action of methyridine is the fact that it is more effective in terms of absolute dose when administered subcutaneously than when administered orally. However, it is also more toxic by subcutaneous injection, so that there is little to choose between the two methods of treatment on the grounds of safety.

In addition to possessing activity against adult nematodes, a good anthelmintic must also be active against the immature forms. The comparative trials indicate that methyridine is at least as good as bephenium against immature nematode infections. It appears that differences in the relative activity of the two drugs against adult worms are reflected in their relative activity against the corresponding immature forms. However, certain exceptions do arise, as illustrated by the trials with 1- and 2-day-old *N. muris* infections; here methyridine appears to be clearly the more effective drug, and it should be noted that the larvae of *N. muris* are actually migrating through the lung at this time. Thus methyridine is able to affect nematode larvae which are not even in the gut. This feature is unlikely to be shared by any of the conventional relatively insoluble anthelmintics which are poorly absorbed by the mammalian host.

The authors are pleased to acknowledge their gratitude to Miss R. M. Potts and Mrs J. Parkinson for their able technical assistance with these studies.

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STUDIES ON THE MODE OF ACTION OF METHYRIDINE

BY

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Methyridine [2-(β-methoxyethyl)pyridine] reached the blood and all regions of the alimentary canal following oral or subcutaneous administration of therapeutic doses of the drug. Concentrations in the lower gut were highest following subcutaneous administration, and there was a close association between blood and intestinal levels suggesting that the drug is excreted into the alimentary canal along its whole length. *In vitro* experiments with intact nematodes (*Ostertagia spp.* from sheep; *Nematospiroides dubius* and *Heterakis spumosa* from mice; and *Nippostrongylus muris* from rats) and with preparations of *Ascaris sp.* have demonstrated that methyridine passes through the cuticle and exerts an "irreversible" paralysing effect on the worms. This action is obtained within 1 hr at concentrations similar to peak levels shown to be present in the alimentary canal following the administration of therapeutic doses. Concentrations of the drug were found to be higher in the abomasum than in the intestine of sheep and yet the anthelmintic activity of methyridine is greatest against intestinal nematodes. No significant differences in the resistance of various nematode species to the drug were demonstrated, but the amount of methyridine entering ascarids was significantly greater at *p*H 8 than at *p*H 5 or 3. In addition, the *in vitro* anthelmintic activity of the compound was greatest in alkaline solutions, which probably accounts for the differences in anthelmintic activity noted in various parts of the alimentary canal. The paralysing effect of methyridine on nematodes is probably due to a neuromuscular block of the decamethonium type, and this can also be produced in the host by over-dosage. It is suggested that nematode tissues are more sensitive to this action than vertebrate tissues.

Experiments in laboratory animals (Broome & Greenhalgh, 1961a and b) and farm animals (Walley, 1961) have demonstrated considerable anthelmintic activity against nematode infections of the gastro-intestinal tract following treatment with the compound methyridine [2-(β-methoxyethyl)pyridine; "Promintic"]. The same reports indicate a number of features which make the drug unique among known anthelmintics. In particular, it is a liquid which is extremely water-soluble and which is most effective when administered subcutaneously. These peculiarities clearly merited this study of its mode of action. In the work described below estimations have been made of the drug concentration in various parts of the body and of the effect of the drug *in vitro* against various nematodes. The results are then correlated in an attempt to show how the *in vivo* anthelmintic activity is achieved.

METHODS

Chemical estimation of methyridine in tissues. All samples were collected and processed for estimation immediately after sacrificing the animals. They were then stored at +5° C until estimations could be made, and these were always completed within seven days of collection. Experiments showed that deterioration was not significant during this storage period.

One ml. samples (blood, gut contents, tissue homogenates, urine, etc.) were diluted with 1 ml. distilled water before precipitating the proteins with 2 ml. 0.3 N barium hydroxide and 2 ml. 5% zinc sulphate. They were then centrifuged at 3,000 rev./min for 5 min and the supernatant was removed. The precipitate was resuspended and washed with 2 ml. distilled water and a second extraction was performed. The two extracts were mixed, filtered through Whatman No. 40 filter paper, and made alkaline with 0.2 ml. 40% sodium hydroxide. Methyridine was obtained from this crude alkaline extract by evaporation *in vacuo* and collected by sublimation on a cold finger (cooled with solid carbon dioxide and acetone). I am indebted to Dr G. A. Snow for suggesting this method of extraction.

After complete evaporation the sublimate was melted from the cold finger, filtered and made acid with 0.2 ml. concentrated hydrochloric acid before measuring the optical density at 260 mμ with a Beckman spectrophotometer using 1 cm quartz cells. The methyridine concentration was then estimated from standard curves previously prepared by extracting graded amounts of the drug from the material in question.

All determinations were carried out in duplicate, and statistical analysis of the results showed the standard error between individual determinations to be ±10 to 15%. The standard error of the average of duplicate determinations is therefore ±7 to 10%. To this must be added errors due to animal variation before interpreting the results.

The analytical method estimates any pyridine compound which volatilizes under alkaline conditions and it is therefore not specific to methyridine *per se*. Studies by Duncan & Scales (1961) indicate that the only methyridine metabolite likely to be estimated by the method is α-picoline. However, extractions from samples containing added α-picoline have shown this compound to be so volatile that little is collected on the cold finger. It is therefore felt that the results provide a reliable indication of methyridine concentration.

Blood and gut concentrations of methyridine in mice. Groups of 20 mice were dosed with the optimum therapeutic dose of methyridine 400 mg/kg orally and subcutaneously (Broome & Greenhalgh, 1961b). After the appropriate time interval all animals were sacrificed and their blood was collected together with material from various regions of the alimentary canal. The samples were pooled to provide sufficient material for the chemical estimations of drug concentration.

Blood and gut concentrations of methyridine in sheep. Blood levels of the drug were followed in groups of two sheep, bled from the jugular vein at hourly intervals after dosing with either 150 or 200 mg/kg given subcutaneously or orally. Blood and gut levels were also determined in groups of two sheep, given the optimum therapeutic dose of 200 mg/kg subcutaneously (Walley, 1961) and sacrificed after the appropriate time interval. Finally, a comparison between blood and gut drug concentrations following oral and subcutaneous administration of 150 mg/kg was made in two sheep.

In vivo *anthelmintic activity*. This was estimated in mice by the method previously described (Broome & Greenhalgh, 1961b).

In vitro *anthelmintic activity*. Serial dilutions of methyridine and some of its metabolites were prepared in simple Ringer solution or in filtered gut contents from untreated sheep. They were placed in 10 cm petri dishes and warmed to 37° C before adding the nematodes *Heterakis spumosa* and *Nematospiroides dubius* (from mice), *Nippostrongylus muris* (from rats) and where possible *Ostertagia spp.* (from sheep). After incubating for the required time interval all worms were examined for motility. In the experiments to determine the speed at which the drug causes "irreversible paralysis" to *H. spumosa* the worms were allowed a 30 min recovery period in fresh Ringer solution before estimating anthelmintic activity by

the percentage immobile worms. Finally, the survival of worms in filtered gut contents taken from sheep 2 hr after dosing with 200 mg/kg of methyridine subcutaneously was examined.

Effect of pH on in vitro *anthelmintic activity. In vitro* survival studies were carried out as previously described but with the serial dilutions of the drug prepared in Ringer solution buffered with citrate-phosphate buffer. At the termination of each experiment the *p*H of all test solutions was checked by means of a glass electrode.

The effect of pH on the uptake of methyridine from solutions by Ascaris sp. *from pigs.* A 2×3 factorial experiment was used to study the uptake of methyridine by *Ascaris sp.* over periods of 3 and 6 hr at *p*H's of 3, 5 and 8 respectively. About 75 g ascarids were mopped with filter-paper to remove excess water and they were then divided into six approximately equal groups on the basis of individual worm size. Each group was randomly assigned to one of the treatments and allowed to equilibrate for 15 min in Ringer solution buffered at the appropriate *p*H. Solutions containing 100 μg/ml. of drug were prepared in Ringer solution buffered at the required *p*H by means of a citrate-phosphate buffer. After equilibration the worms were transferred to their respective methyridine solutions allowing 1 g/4 ml. solution. Duplicate 1 ml. samples were immediately taken from all solutions containing ascarids and also from identical control solutions with no ascarids. After incubation for 3 hr at 37° C the appropriate tubes and their respective controls were sampled in duplicate. In addition each worm was weighed and homogenized individually. Similar samples were collected from the remaining tubes and their controls after 6 hr incubation at 37° C. Chemical estimations on the ascarid homogenates were carried out by the method previously outlined. The *p*H of all solutions was checked at the end of the experiment by means of a glass electrode. The concentration of drug in the samples from all solutions was estimated from optical density measurements made after diluting with 4 ml. N/100 hydrochloric acid to remove any effect of *p*H.

Oxygen consumption of ascarids. The effect of methyridine on the oxygen consumption of pig ascarids was examined with the Warburg manometric technique outlined by Lasser (1944). These experiments were kindly performed by Dr J. F. Ryley. Comparisons were made between results obtained with whole worms and worms divided into 0.5 in. segments. Any effect of delayed drug absorption by the worms was thereby examined.

Neuromuscular blocking activity. These studies were also carried out with pig ascarids using isolated neuromuscular preparations as described by Baldwin (1943). In comparative experiments with other compounds known to affect cholinergic nerve endings or acetylcholine, a modification of the exposed neuromuscular preparation (Baldwin & Moyle, 1947) was used. This involved making a single slit along the lateral line of each preparation to allow the entry of compounds otherwise unable to penetrate the cuticle.

RESULTS

The distribution of methyridine in the blood and alimentary tract of mice and sheep

Blood and gut methyridine concentrations in mice. Analytical results of blood and gut contents taken from mice, given a single therapeutic dose of methyridine 400 mg/kg subcutaneously or orally, are summarized in Fig. 1. This shows that the drug is absorbed and metabolized rapidly with maximum blood concentrations 1 hr after dosing and that little remains 5 hr later. (Excretion in mice amounts to only 1 to 2% of the dose during the 48 hr following administration.) There appears to be a close similarity between drug concentrations in the blood, small intestine, caecum and large intestine. The stomach, however, has much higher and more persistent levels than the other regions of the body.

Drug concentrations in the blood, small intestine, caecum and large intestine are higher following subcutaneous administration than following oral administration.

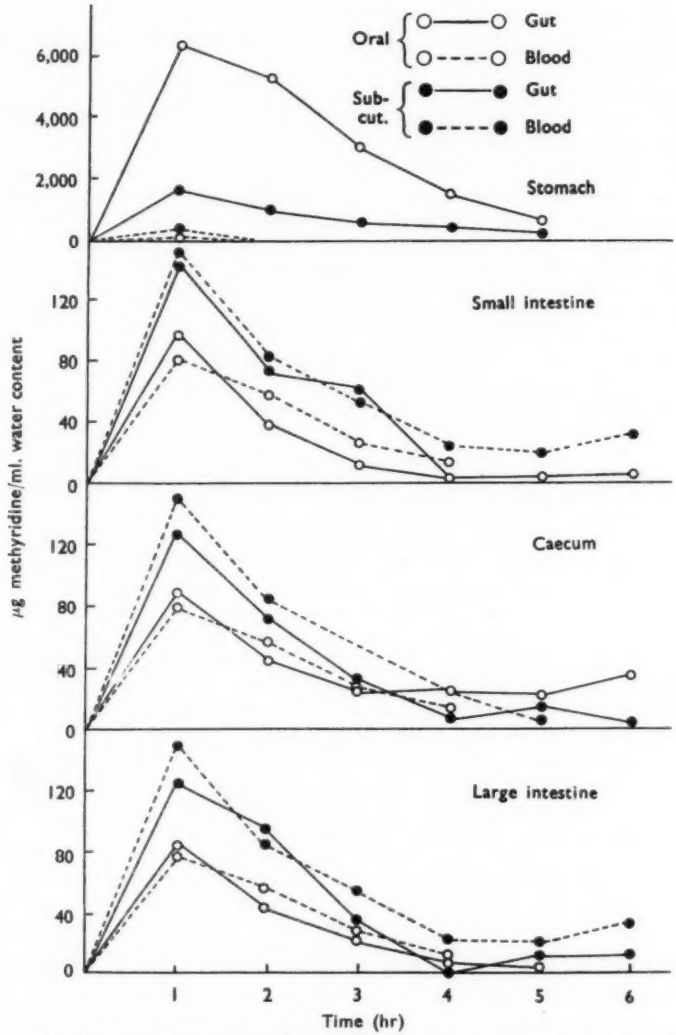

Fig. 1. Blood and gut concentrations of methyridine in mice following subcutaneous and oral dosing with 400 mg drug/kg.

but stomach levels are greater when the drug is given orally. Examination of the relationship between maximum blood levels following oral, and subcutaneous, administrations indicates a ratio of 1/1.67 respectively.

Blood and gut methyridine concentrations in sheep. Fig. 2 shows the results of analysis of blood and gut material collected from a total of six sheep killed after receiving a single dose of 200 mg/kg subcutaneously. As in mice, it is

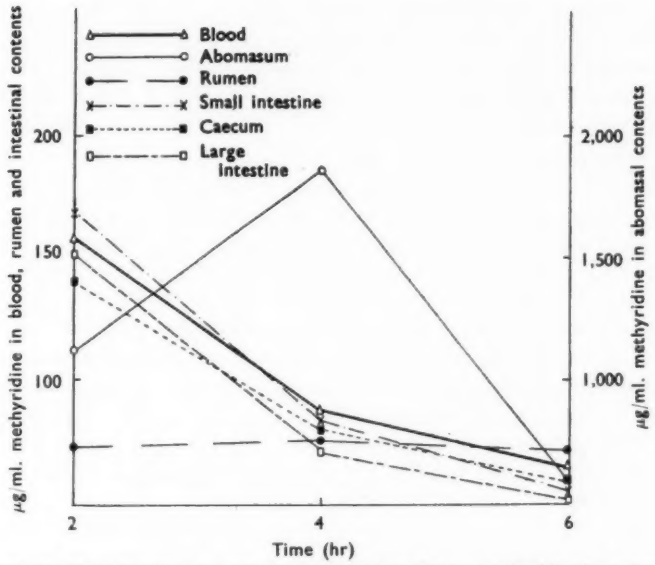

Fig. 2. Analysis of blood and gut contents from sheep given 200 mg methyridine/kg subcutaneously.

apparent that methyridine is absorbed and metabolized rapidly and maximum blood levels are attained approximately 2 hr after dosing (Fig. 3). The drug concentration in all regions of the alimentary canal except the abomasum is closely related to the corresponding blood level. Levels in the abomasum are much higher and more persistent than elsewhere. Thus, the distribution of methyridine along the alimentary tract is similar in both mice and sheep. The effect of oral and subcutaneous dosing on blood concentrations is shown in Fig. 3. The ratio between

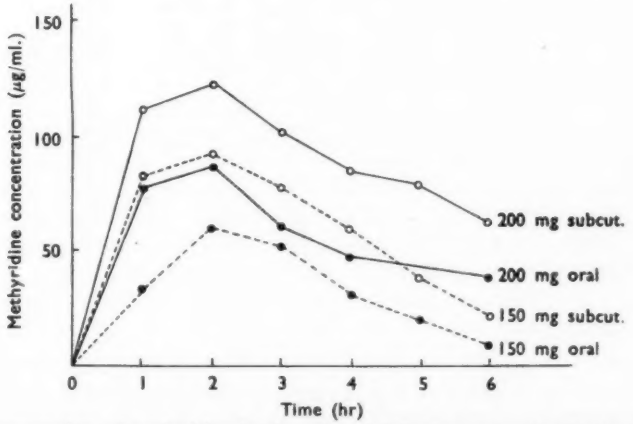

Fig. 3. Concentration of methyridine in sheep blood following oral and subcutaneous dosing.

maximum blood levels is 1/1.47 and that for the areas under the curves is 1/1.42, for oral and subcutaneous dosing respectively. Analysis of blood and gut contents from the sheep, treated with 150 mg/kg orally or subcutaneously, indicated that these differences in blood level are probably reflected in all regions of the alimentary canal except the rumen and abomasum.

Having shown that methyridine reaches the gut in most regions, it became important to know whether physiological concentrations of the drug had any anthelmintic effect on nematodes *in vitro*. In addition the anthelmintic activity of its most likely metabolites was examined both *in vivo* and *in vitro*.

The anthelmintic activity of methyridine and some of its metabolites

The *in vitro* survival studies were disappointing in that motility—the end point for estimating anthelmintic activity—could not be assessed with sufficient precision to allow an accurate quantitative evaluation of the results. Figures quoted in the tables should only be regarded as a qualitative guide to the relative effectiveness of compounds tested.

In vivo *and* in vitro *anthelmintic activity of methyridine and some of its metabolites*. The results from these studies are presented in Table 1, from which

TABLE 1

THE ANTHELMINTIC ACTIVITY OF METHYRIDINE AND SOME OF ITS POSSIBLE METABOLITES

Anthelmintic test:	Minimum drug concentrations "killing" worms at 2 hr *in vitro* (mg/ml.)				*In vivo* mouse test (using maximum tolerated dose)		
Nematode species:	*H. spumosa*	*N. muris*	*N. dubius*	*Ostertagia spp.*	*H. spumosa*	*N. muris*	*N. dubius*
Compound							
Methyridine	0·06	0·06	0·06	0·06	Active	Active	Active
2-Pyridyl acetic acid hydrochloride	1	10	10	10	No action	No action	No action
Pyridyl ethanol	10	10	10	10	No action	No action	No action
α-Picolinic acid	0·5	40	40	40	No action	No action	No action
α-Picoline	10	10	10	10	No action	No action	No action
Formaldehyde	1	10	10	No test	No action	No action	No action
Formic acid	1	10	10	No test	No action	No action	No action

it is clear that methyridine possesses more anthelmintic activity than its metabolites (identified by Duncan & Scales, 1961) both *in vivo* and *in vitro*. Furthermore, the concentrations of methyridine required for *in vitro* activity are in the same range as those previously shown to be present in the gut contents of animals treated with the drug. The similarity in the results with all the nematode species studied suggests very little species variation in susceptibility to the drug. Here it may be noted that the apparent susceptibility of *H. spumosa* to α-picolinic acid and 2-pyridyl-acetic acid hydrochloride probably arises indirectly as a result of the low *p*H of these solutions.

Experiments in which filtered gut contents from untreated sheep served as the diluent indicated that they did not diminish the *in vitro* activity of methyridine. Tests with material obtained 2 hr after dosing sheep with 200 mg/kg subcutaneously indicated activity similar to that anticipated from analytical results.

Having shown that the peak drug concentrations in the gut contents of treated animals are sufficient to affect nematodes *in vitro*, it was necessary to find out if they are also sufficiently prolonged to remove the worms. Studies were therefore undertaken to find the time required by solutions of various concentrations to produce " irreversible paralysis " in *H. spumosa.*

The onset of methyridine-induced paralysis in the nematode H. spumosa. " Irreversible paralysis " is defined here to mean paralysis which could not be reversed by a 30-min recovery period in fresh Ringer solution. The outstanding feature of these results was the rapidity with which methyridine affected the test nematodes. Thus all worms in contact with solutions of peak physiological concentrations were irreversibly paralysed after approximately 1 hr. In more dilute solutions those worms, which were going to succumb, did so within 2 hr ; after which there was no further effect over a 5-hr test period.

These results indicate that the concentrations of drug in the alimentary canal of mice and sheep following therapeutic doses are sufficiently prolonged to account for its *in vivo* anthelmintic activity. The results obtained by Walley (1961) indicate that it is less efficient in the abomasum than in the small intestine of sheep despite the fact that concentrations were higher in the abomasum. The effect of a number of factors which might modify its activity in the abomasum was therefore examined in an attempt to provide an explanation for these apparently anomalous results.

The effect of p*H on the* in vitro *anthelmintic activity of methyridine.* Preliminary studies with Ringer solution of varying *p*H showed the nematode *N. muris* to be the most suitable for these studies, as it survived the widest range of *p*H. Table 2

TABLE 2

THE EFFECT OF *p*H ON THE *IN VITRO* ANTHELMINTIC ACTIVITY OF METHYRIDINE AGAINST *N. MURIS*

N.A.=No anthelmintic activity, Sl.A.=slight anthelmintic activity, A=anthelmintic activity, estimated at 2 hr

Drug concentration	*p*H				
	3	4	5	7	8
1,000 μg/ml.	N.A.	Sl.A.	A	A	A
200 μg/ml.	N.A.	Sl.A.	A	A	A
100 μg/ml.	N.A.	N.A.	A	A	A
50 μg/ml.	N.A.	N.A.	Sl.A.	A	A
25 μg/ml.	N.A.	N.A.	N.A.	N.A.	N.A.

shows that the strongest solution of drug (1,000 μg/ml.) had no effect on *N. muris* at *p*H 3, but as the solutions became more alkaline activity increased so that 50 μg/ml. paralysed worms at *p*H 7 and 8. A similar effect was seen with *Ascaris sp.* from pigs and to a more limited extent with *Ostertagia spp.* (which do not tolerate the more alkaline conditions). No effect of *p*H was seen with *H. spumosa* because the worms died in solutions of low *p*H as a result of acidity *per se.*

The uptake by pig ascarids of methyridine from solutions of varying p*H.* Before analysing the uptake of drug by *Ascaris sp.* from the various test solutions, adjust-

ments were made for any change in drug concentration which occurred in corresponding controls. In addition, the relationship between individual worm size and the methyridine concentration of appropriate homogenates was examined. No statistically significant effect of worm size was evident, and it was therefore unnecessary to make adjustments for any variation in individual worm weights between treatments.

Since ascarids do not appear to metabolize methyridine (Duncan, unpublished data), it is considered that Fig. 4 shows the effect of *p*H on the ability of the drug

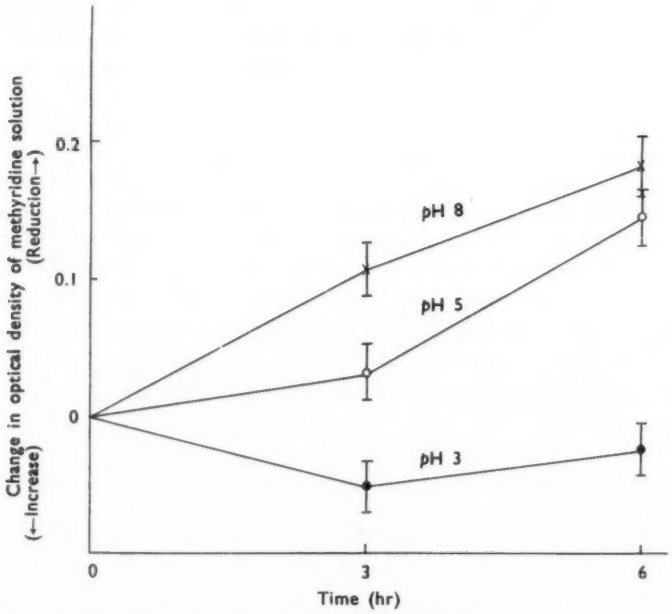

Fig. 4. Uptake of methyridine from solutions of differing *p*H by *Ascaris sp.* from pigs.

to enter the nematodes. A definite effect of *p*H is shown by more drug entering the worms maintained under alkaline conditions. The apparent increase in the methyridine content of solutions at *p*H 3 is not statistically significant. There appears to be a constant relationship between drug uptake and time.

Chemical analysis of worm homogenates showed that the concentration of methyridine within the worms after 6 hr at *p*H 8 was approaching that in the outside solution. Thus it appears that the drug passes into nematodes according to the concentration gradient and that preferential absorption does not occur.

The effect of methyridine on parasitic nematodes

Another important aspect of this study on the mode of action of methyridine is a consideration of the way in which the drug actually affects the parasitic

nematodes. The investigations reported below concern its action on aerobic metabolism and neuromuscular activity in *Ascaris sp.* from pigs.

Studies on the aerobic metabolism of Ascaris sp. The results of this experiment are presented in Table 3, which shows that methyridine does not significantly inhibit the aerobic metabolism of this nematode.

TABLE 3

THE EFFECT OF METHYRIDINE ON THE OXYGEN CONSUMPTION OF *ASCARIS SP.*

Treatment (methyridine/ml.)	μl. oxygen utilized by 1.1 g worm tissue/hr	
	Whole worms	Cut worms
Control	126	111
10 mg	100	103
3·3 mg	121	99
1.1 mg	135	92

Studies on neuromuscular inhibition. The effect of the drug on segments of ascarid worms with the cuticle intact is illustrated in Fig. 5. This shows that extremely rapid paralysis with contraction follows the addition of small amounts of drug to the tissue bath. A comparison of the methyridine-effect with that of

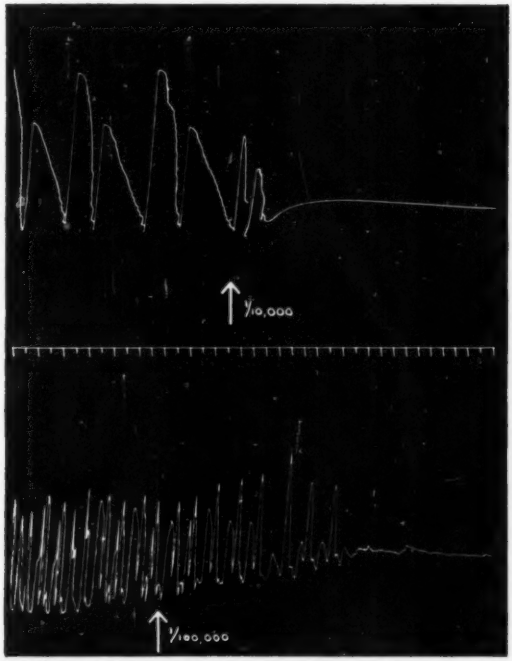

Fig. 5. Effect of methyridine on intact segments of *Ascaris sp.* Time marker 30 sec.

D-tubocurarine using slit preparations is presented in Table 4. For completeness the results of Norton & De Beer (1957) with piperazine and decamethonium are also included in the table.

TABLE 4

COMPARISON OF NEUROMUSCULAR BLOCKING AGENTS IN SPLIT *ASCARIS* PREPARATIONS

The results for piperazine and decamethonium were taken from Norton & De Beer (1957)

	Effective concentration	Effect on *Ascaris* preparations	Effect of acetylcholine chloride (10 μg/ml.)
Tubocurarine chloride	5 μg/ml.	Flaccid paralysis	Antagonism
Methyridine	10 μg/ml.	Partially contracted paralysis	No effect
Piperazine citrate	250 μg/ml.	Flaccid paralysis	Antagonism
Decamethonium bromide	40 μg/ml.	Contracted paralysis	No effect

DISCUSSION

The chemical analyses of blood and gut contents from mice and sheep show that methyridine behaves very similarly in both species. These will therefore be considered together whenever possible. It is clear from Figs. 1 and 2 and Table 1 that when the drug is given in therapeutic doses administered either orally or subcutaneously it reaches all regions of the alimentary canal in concentrations sufficient to affect a variety of nematodes *in vitro*. The period of maximum gut concentration is short, but it appears to be quite sufficient to cause "irreversible paralysis" to *H. spumosa*.

Anthelmintic studies in laboratory animals (Broome & Greenhalgh, 1961b) have shown that the drug is a more efficient anthelmintic when administered subcutaneously than when given orally. This is in accordance with the results of these chemical analyses, which show greater drug concentrations in the gut contents of animals treated subcutaneously (except in the stomach, rumen and abomasum). The alternative hypothesis that the drug is metabolized to an active anthelmintic seems unlikely, because all the metabolites indicated by Duncan & Scales (1961) are less active (Table 1). In addition, the *in vitro* anthelmintic activity of gut contents collected from sheep 2 hr after a subcutaneous dose of 200 mg/kg of drug is no greater than would be expected from the chemical analysis.

There is a close association between drug concentrations in the blood and contents of all parts of the alimentary canal except the stomach and abomasum. This seems to indicate the drug is absorbed into the blood following both oral and subcutaneous dosing, and is subsequently re-excreted all along the lower regions of the alimentary canal. As metabolism proceeds and blood levels fall, the drug is re-absorbed and gut levels fall. This hypothesis is supported by the results (Raventós & Broome, unpublished data) of studies in rats showing that methyridine readily passes from the blood to the gut, and vice versa, according to the concentration gradient, in all regions of the alimentary canal except the stomach.

This mechanism, by which the compound reaches the intestinal regions of the digestive system, appears unique among known anthelmintics, and it helps to explain

certain uncommon features associated with the drug's activity. Firstly, its concentration and its persistence in all regions of the lower gut appear to depend largely on the level in the blood rather than on the rate at which food passes through the alimentary canal. Furthermore, it seems to pass from the blood to the gut along the whole length of the alimentary canal. This allows the drug intimate contact with all worm species, even if they are embedded in the intestinal mucosa or lying beneath the mucous lining of the digestive tract. The worms therefore receive no protection from their varying habitats, a factor which undoubtedly contributes to the broad spectrum of anthelmintic activity following treatment with methyridine.

The analytical results of material from the stomach (mice) and abomasum (sheep) show that their drug concentrations do not follow this "generalized intestinal plan." Thus it can be seen from Figs. 1 and 2 that the concentrations in these regions are extremely high and bear little or no relationship to the corresponding blood levels. Using rats as experimental animals Raventós & Broome (unpublished data) have shown that methyridine passes rapidly into the stomach against the concentration gradient and that conversely absorption from the stomach is extremely slow. This effect appears to be related to the acidic secretions of these organs which presumably cause the ionization of the basic methyridine molecule. The latter is thereby effectively removed from any equilibrium controlling the passage of drug into the gut.

Although there is a high concentration of drug in the abomasum, Walley (1961) has shown it to be less effective against abomasal than intestinal parasites. The data of Walley also indicate that if abomasal nematodes inhabit the small intestine they are just as susceptible to treatment as are normal intestinal species. This suggests that the relatively poor anthelmintic effect reported by Walley against abomasal parasites is not the result of a differential species susceptibility to the drug. The effect of *p*H on the *in vitro* anthelmintic activity (Table 2) appears to offer a pharmacological explanation of Walley's results. As *p*H is lowered the activity decreases until at a *p*H of 3 the drug is ineffective at physiological concentrations. Abomasal *p*H lies between 3.5 and 4.5 according to Masson & Phillipson (1952). The most likely reason for this *p*H effect is that the unionized molecule is the active component since it alone can penetrate the lipid barrier of the nematode cuticle. Acidity favours ionization of the molecule and thus decreases its lipid solubility which slows down its entry into the worms. When the *p*H falls below approximately *p*H 3 the molecule is completely ionized and does not enter the worm to produce its toxic effect. Thorp (unpublished data) estimates the pK_a of methyridine as approximately 5.5.

The experimental results illustrated in Fig. 4 show that acidity does, in fact, significantly reduce the uptake of methyridine by nematodes of the genus *Ascaris*. Whether this is the only reason for the relative inefficiency of the drug in the abomasum can only be conclusively proved by *in vivo* experiments involving modifications of abomasal *p*H.

The final aspect of this work concerns the manner in which the drug produces its toxic effect in parasitic nematodes. The chemical similarity between methyridine and nicotinic acid or nicotinamide suggested the drug might affect aerobic metabolism. However, Warburg studies (Table 3) revealed no evidence to support this hypothesis.

Nevertheless the results were interesting because they showed the nematodes were still " alive " even though completely paralysed.

Investigations into the nature of methyridine-induced paralysis indicated a type of neuromuscular inhibition which cannot be antagonized by acetylcholine. This suggests a block of the decamethonium type which had previously been demonstrated by my colleague, Dr. J. Raventós, to be caused by the drug in vertebrates. Despite the similar pharmacological activity of methyridine in the host and its nematode parasites, the drug can be used as a safe and effective therapeutic agent (Broome & Greenhalgh, 1961 ; Walley, 1961). Since similar amounts of methyridine appear to reach the tissues of both host and parasite, it seems likely that the usefulness of the drug must depend on the greater sensitivity of nematode than vertebrate tissue to its action. Indirect evidence in support of this hypothesis is available because concentrations of 10 μg/ml. will completely paralyse intact ascarid segments (Fig. 5) whilst blood levels of over 100 μg/ml. have not caused fatalities in mice, rats and sheep. The therapeutic usefulness of the drug therefore appears to depend on quantitative differences between nematode and vertebrate neuromuscular transmission.

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EFFECTS OF COCAINE AND ANTIDEPRESSANT DRUGS ON THE NICTITATING MEMBRANE OF THE CAT

BY

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Cocaine, imipramine and pipradol potentiated the contractions to adrenaline and noradrenaline, but not to tyramine, on the nictitating membrane of the spinal cat. Pheniprazine and dexamphetamine potentiated the responses to adrenaline, noradrenaline and tyramine, whereas nialamide only potentiated the response to tyramine. Potentiation of the response to stimulation of either the preganglionic or the postganglionic sympathetic nerve trunks was observed with imipramine, pipradol, pheniprazine and dexamphetamine. Only dexamphetamine and pheniprazine caused substantial contractions of the membrane when the preganglionic nerve was cut (acutely decentralized), or when the superior cervical ganglion was removed (acutely denervated). Cocaine produced contractions of the innervated but not of the acutely decentralized membrane. The significance of the peripheral effects of these antidepressant drugs in relation to their central actions is discussed.

Several workers, for example, Rothballer (1957), Costa & Zetler (1959), Sigg (1959), and Maxwell, Sylwestrowicz, Plummer, Povalski & Schneider (1960), have speculated on the possible relationship between the central actions of many stimulant drugs and their effects on the peripheral actions of adrenaline. In the present investigation five antidepressant drugs (imipramine, pipradol, pheniprazine, nialamide and dexamphetamine), which have all been used with varying degrees of success in the treatment of depressive illness (Rees, 1960), and cocaine, have been compared for their ability to modify the actions of adrenaline, noradrenaline, tyramine and sympathetic nerve stimulation on the nictitating membrane of the spinal cat. Tyramine was included since it is thought to be metabolized *in vivo* by monoamine oxidase (Corne & Graham, 1957), and the effect of tyramine on the membrane is known to be potentiated by iproniazid, which is an inhibitor of the enzyme (Griesemer, Barsky, Dragstedt, Wells & Zeller, 1953).

It was hoped that the accumulation of the results of this investigation might show certain similarities in some of the peripheral actions of these antidepressant compounds, and thus contribute to an understanding of their mechanism of action.

METHODS

Preparation of spinal cat. The spinal cord was cut at the level of the second cervical vertebra and the brain was destroyed under ether anaesthesia (Burn, 1952). Seventy-five such preparations were used.

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Nictitating membrane. The contractions of the nictitating membrane were recorded by a frontal writing lever, writing on a smoked drum. The contractions were magnified 10 times. Records of the size of the contractions were those measured on the drum. Usually the membrane was acutely decentralized by cutting the preganglionic nerve, but in some experiments the innervation was left intact; when the postganglionic nerve was stimulated the superior cervical ganglion was removed at the beginning of the experiment (acute denervation). An interval of about 1 hr was then allowed to elapse before continuing. Injections were made into the femoral vein. Blood pressure was recorded with a mercury manometer from either a carotid or a femoral artery.

Effect of drugs. The test doses of adrenaline and noradrenaline ranged from 1 to 40 μg, and produced contractions of 2 to 15 mm of the decentralized membrane. Tyramine was given in a dose of 0.25 to 2 mg and gave contractions of 1 to 40 mm. Adrenaline and noradrenaline were injected alternately at intervals of about 5 to 10 min, and tyramine was injected at intervals of 15 to 60 min. However, in a few experiments it was possible to inject tyramine at 10 min intervals without a reduction in sensitivity occurring.

One estimate of the degree of potentiation was made by determining the ratio of the doses producing the same size of contraction before and after the test drug. In most instances the effects of single doses of the antidepressant drugs were observed until a maximum potentiation had been obtained. Another estimate of potentiation was determined from the duration of the contractions, which was expressed as the half-life, that is, the time to 50% recovery. Then the potentiation of the duration was expressed as the ratio of the half-life after the test drug to the half-life of the control response.

Sympathetic nerve stimulation. In order to investigate the potentiating effect of drugs on sympathetic nerve stimulation, the cut preganglionic cervical sympathetic nerve was stimulated submaximally (Trendelenburg, 1959), so as to give contractions between 20% and 60% of the maximum. In some experiments the contractions of the contralateral membrane to stimulation of the stump of the postganglionic nerve were recorded as well. In these experiments the superior cervical ganglion on one side was removed. Square-wave stimuli, of 0.5 msec duration, were delivered to the nerve at a frequency of 10/sec for 15 sec every 2 min. Since it was difficult to maintain constant responses to submaximal stimulation over long periods, the test substances were injected when the contractions remained steady, or were declining slightly for about 10 min, and only those effects which were observed soon after the injection were considered to be significant.

Drugs. The drugs used were cocaine, imipramine, pheniprazine, pipradol, adrenaline, and tyramine as the hydrochlorides, dexamphetamine sulphate, noradrenaline bitartrate, 5-hydroxytryptamine creatinine sulphate, hexamethonium bromide, pyrogallol and nialamide. The doses are expressed in terms of the salt, except for nialamide and pyrogallol, which were injected as the pure compounds dissolved in water.

RESULTS

The main results on all the compounds are summarized in Fig. 1, and in Table 1 these results have been expressed quantitatively where this was possible. Whereas it was often feasible to determine the degree of potentiation of the height of contraction for adrenaline, as described in the methods, it was not often possible for noradrenaline since the nictitating membrane was very insensitive to this substance, even though adrenaline and noradrenaline were roughly equipotent in causing pressor responses: in 17 experiments the average control contraction to 20 μg of noradrenaline was only 4 ± 1.7 mm (standard error), in contrast to adrenaline (10 μg) which produced an average contraction of 24 ± 5.4 mm.

The half-life of the contractions to adrenaline and noradrenaline ranged from 0.5 to 1.5 min in different experiments, and did not vary by more than a factor of

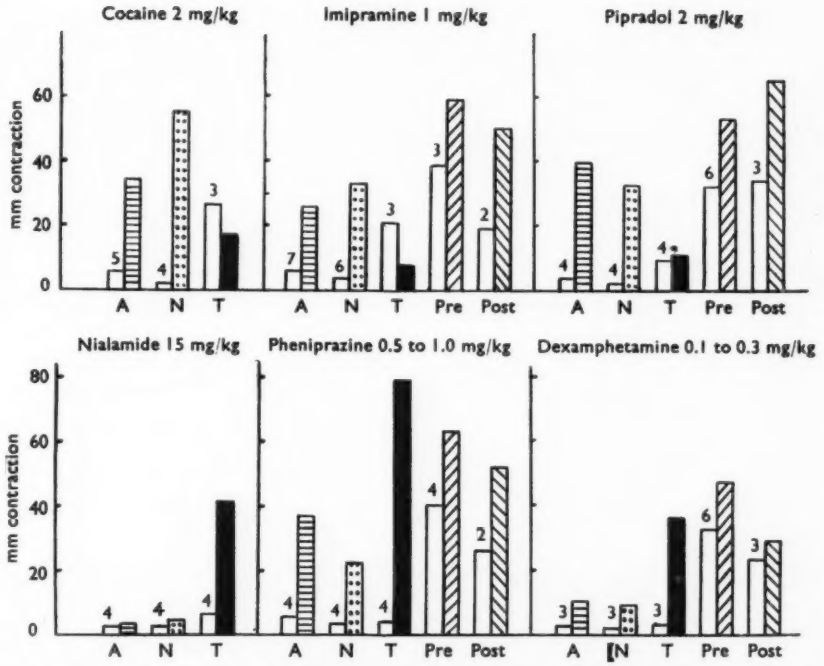


Fig. 1. Spinal cats. Effects of cocaine and antidepressant drugs on contractions of the acutely decentralized nictitating membrane to adrenaline (A), noradrenaline (N), tyramine (T) and submaximal stimulation of the preganglionic nerve (Pre), and on contractions of the acutely denervated membrane to submaximal stimulation of the postganglionic nerve (Post). Control observations are indicated by the blank columns, and the number of experiments from which the average heights of contraction were calculated are shown above the control columns. A dose of 1 mg/kg of pheniprazine was used in experiments on A, N and T, and 0·5 mg/kg was used in experiments on Pre and Post. The dose of dexamphetamine varied from 0.1 to 0.3 mg/kg in different experiments.

two over an 8-fold increase in the dose in any given experiment. The half-life of the contractions to tyramine ranged from 1 to 5 min and also did not vary by more than a factor or two on increasing the dose. Thus in Table 1 a potentiation of the duration of the contractions of less than two cannot be considered to be significant.

Cocaine. The intravenous injection of 0.25 to 2 mg/kg of cocaine produced only a small contraction (2 ± 0.7 mm) of the acutely decentralized membrane (11 experiments). This result is in agreement with the observation made by Trendelenburg (1959). He found that cocaine (0.2 to 6.2 mg/kg) produced a negligible contraction of the acutely decentralized membrane in the spinal cat. In contrast to this small effect, there was often a much larger contraction of the normally innervated membrane, although the response was extremely variable (Table 2). The size of this contraction ranged from 2 to 110 mm, and the duration varied from 3 min to more than 45 min.

TABLE 1

EFFECT OF COCAINE AND ANTIDEPRESSANT DRUGS ON CONTRACTIONS TO SYMPATHOMIMETIC AMINES ON ACUTELY DECENTRALIZED NICTITATING MEMBRANE OF THE SPINAL CAT

The numbers of experiments are shown in parentheses. Potentiation was calculated as the ratio of the doses producing the same size of contraction before and after the test drug

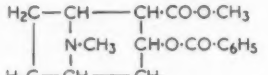
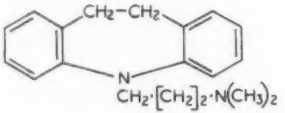
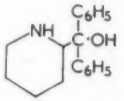
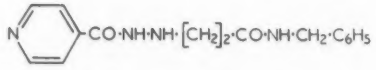
	Formula	Total no. of cats	Dose mg/kg injected intravenously	Adrenaline: Average potentiation of height	Adrenaline: Average potentiation of duration	Noradrenaline: Average potentiation of height	Noradrenaline: Average potentiation of duration	Tyramine: Average potentiation of height	Tyramine: Average potentiation of duration	Direct effect on membrane (average contraction in mm)
Cocaine		3	0·5	1·4 (1)	1·0 (3)	—	1·4 (3)	0·8 (1)	1·0 (1)	1·0 (1)
		2	1·0	3·5 (1)	1·0 (1)	—	1·0 (1)	0·5 (1)	1·0 (1)	0·0 (2)
		6	2·0	2·8 (2)	1·6 (5)	—	2·0 (4)	0·6 (3)	1·0 (3)	0·0 (6)
Imipramine		11	1·0	2·4 (5)	4·0 (7)	>2 (1)	1·3 (4)	0·5 (4)	1·0 (4)	0 (11)
Pipradol		4	2·0	2·0 (2)	1·6 (4)	2·5 (2)	3·3 (4)	1·1 (3)	1·0 (4)	0 (4)
Nialamide		4	15·0	1·0 (4)	1·0 (4)	1·0 (4)	1·0 (4)	2·0 (1)	>15·0 (4)	0 (4)
Pheniprazine	$C_6H_5.CH_2.CH(CH_3).NH.NH_2$	7	1·0	3·5 (3)	1·9 (4)	3 (4)	1·9 (4)	>5 (4)	7·5 (4)	23 (7)
Dexamphetamine	$C_6H_5.CH_2.CH(CH_3).NH_2$	4	0·05	1·8 (3)	1·5 (3)	1·7 (3)	1·3 (3)	>2 (1)	1·4 (3)	9 (4)
		4	0·1 to 0·3	2·0 (1)	1·5 (3)	—	1·3 (3)	>2 (1)	1·4 (3)	16 (4)

TABLE 2

COMPARATIVE EFFECTS OF COCAINE ON THE NORMALLY INNERVATED AND ON THE ACUTELY DECENTRALIZED NICTITATING MEMBRANES OF THE SPINAL CAT

Duration of contraction is expressed as the half-life, that is, time to 50% recovery

Intravenous dose of cocaine mg/kg	Normally innervated membrane			Acutely decentralized membrane		
	Average height of contraction (mm)	Range (mm)	No. of experiments	Average height of contraction (mm)	Range (mm)	No. of experiments
0·25	6	0 to 12	2	0	—	1
0·5	30	0 to 95	7	1·8	0 to 7	6
1·0	5	0 to 10	3	0·8	0 to 5	6
2·0	35	5 to 110	6	0·5	0 to 4	8
	Average duration of contraction (min)	Range (min)		Average duration of contraction (min)	Range (min)	
0·25	8	—	1	—	—	—
0·5	>12	3 to >45	6	13	3 to 24	2
1·0	>9	7 to >12	2	5	—	1
2·0	>20	3 to >30	6	3	—	1

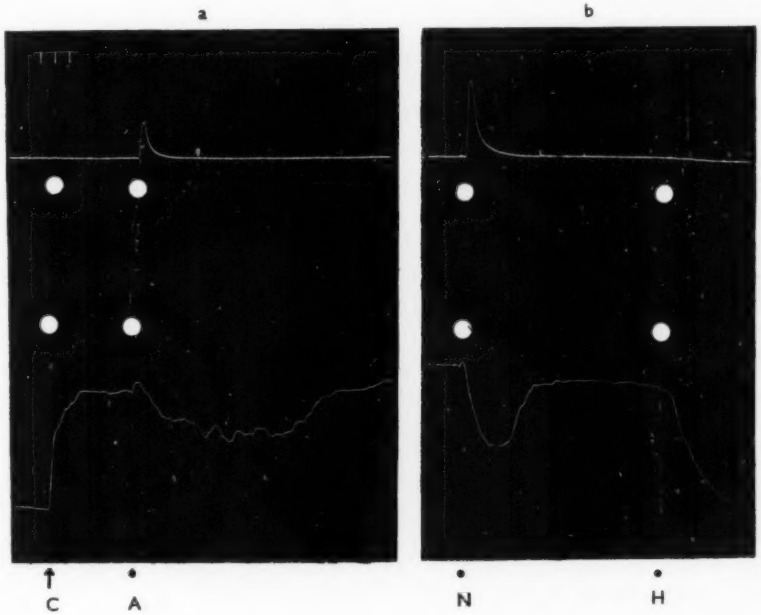


Fig. 2. Spinal cat. Records of contractions of nictitating membrane. Above: acutely decentralized; and below: normally innervated. C=cocaine, 0.5 mg/kg; A=adrenaline, 2 μg; N=noradrenaline, 10 μg; and H=hexamethonium 0.15 mg/kg. Time scale=1 min. Interval of 6 min between *a* and *b*. All injections made intravenously.

The record shown in Fig. 2 is from an experiment in which records were obtained from both the normally innervated and the acutely decentralized membranes. There was a 40 mm contraction of the normally innervated membrane, produced by a second dose of cocaine of 0.5 mg/kg (the first dose produced a larger contraction, 80 mm, but it was of shorter duration). The injection of 2 μg of adrenaline or 10 μg of noradrenaline, which initially produced small contractions (2 to 4 mm) of both membranes, now produced a partial relaxation of the innervated membrane. The membrane also relaxed after hexamethonium. When the effect of hexamethonium had partly worn off, after 12 min, cutting the preganglionic nerve again caused the membrane to relax ; this effect of cutting the preganglionic nerve was seen in two other experiments. The relaxation produced by adrenaline and noradrenaline was probably due to ganglionic blockade, since adrenaline is known to inhibit transmission through sympathetic ganglia (Marrazzi, 1939 ; Bülbring, 1944 ; Matthews, 1956).

In two other experiments adrenaline and noradrenaline (0.5 to 20 μg) produced a partial relaxation of the membrane. The maximum effect observed was an 80% reduction in the height of the contraction.

It was thought that one reason for the failure consistently to reproduce the prolonged contractions of innervated membrane after injections of cocaine may have been due to the time relationship between preparation of the cat and the injection of cocaine. Therefore, two experiments were carried out in which cocaine was injected as soon as possible after cutting the spinal cord and setting up the preparation. In both experiments cocaine (0.5 mg/kg) caused large contractions (30 to 70 mm) of both the acutely decentralized and the normally innervated membranes. However, in one of these experiments the contraction of the innervated membrane was of longer duration than that of the decentralized membrane. Since these large contractions of the decentralized membrane were not observed in the 11 experiments in which the usual 1 hr was allowed to elapse before starting the experiment, it is thought that these contractions may be due to the potentiating effects of cocaine on the circulating catecholamines, released as the result of the ether anaesthesia (Sollmann, 1957) or the trauma from cutting the spinal cord.

Cocaine potentiated the effects of adrenaline and noradrenaline on the decentralized membrane (Fig. 1 and Table 1) ; the maximum potentiation occurred within 10 min. The effect of tyramine, however, was reduced. These results are in agreement with Trendelenburg (1959), who made similar observations on the potentiating effects of cocaine in the spinal cat. He also showed that the contractions produced by submaximal stimulation of either the preganglionic or the postganglionic nerves were increased by cocaine.

Imipramine. Imipramine, in a dose of 1 mg/kg, had no effect on the tone of the acutely decentralized membrane (Table 1). Even higher doses (2 to 4 mg/kg) produced only a small contraction (15 mm) in one out of seven experiments. On the normally innervated membrane doses of 1 to 4 mg/kg had no effect (4 experiments) except in one experiment where a small contraction (10 mm) was obtained.

The effects of imipramine on the responses of the acutely decentralized membrane to adrenaline, noradrenaline and tyramine (Figs. 1 and 3) resembled the effects

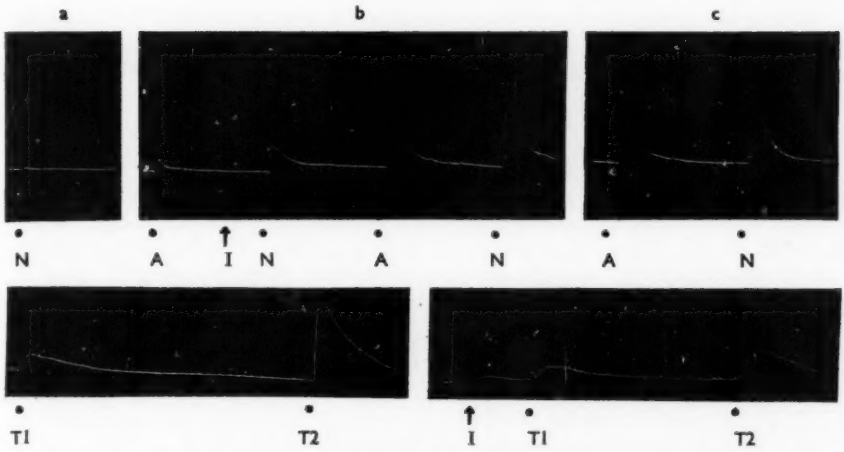


Fig. 3. Spinal cats. Records of contractions of acutely decentralized membranes in two experiments. I=imipramine, 1 mg/kg. Above: N=noradrenaline, 16 μg; A=adrenaline, 4 μg. Interval of 10 min between *a* and *b*, and 60 min between *b* and *c*. Below: T1=tyramine, 1 mg; T2=tyramine, 2 mg; 20 min interval between records. Time scale=1 min. Intravenous injections.

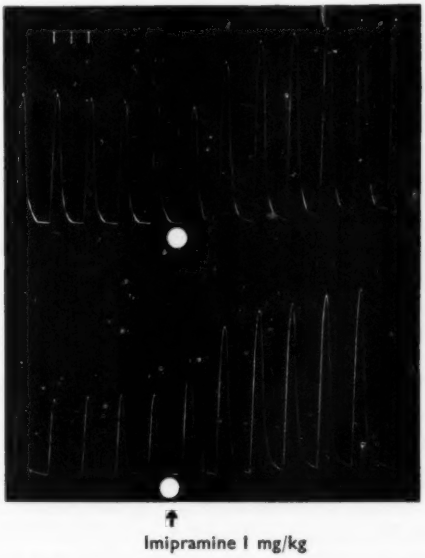


Fig. 4. Spinal cat. Records of contractions of nictitating membrane to submaximal sympathetic nerve stimulation. Above: acutely decentralized membrane (preganglionic stimulation). Below: acutely denervated membrane (postganglionic stimulation). At O imipramine 1 mg/kg was injected intravenously.

obtained with cocaine. As with cocaine, the effects of imipramine were maximal within 10 min of the injection. Fig. 4 illustrates the potentiating effect of imipramine on submaximal stimulation of the preganglionic and postganglionic trunks of the cervical sympathetic nerve. Trendelenburg (1959) showed that cocaine also potentiated the effect of preganglionic and postganglionic stimulation of the cervical sympathetic nerve in the spinal cat.

In view of the similarity between the effects of imipramine and cocaine, it was of interest to see whether imipramine would antagonize the contraction due to amphetamine, since cocaine is known to have this property (Fleckenstein & Stöckle, 1955). It was found (2 experiments) that imipramine (1 mg/kg) caused an immediate relaxation of both the decentralized and the denervated membranes, which were made to contract by an injection of dexamphetamine (0.4 mg/kg).

Furchgott (1955) has suggested that iproniazid, which is an inhibitor of monoamine oxidase, can be used as a tool to study the mechanism of the potentiating effect of cocaine on responses to catecholamines. He showed that cocaine potentiated the contractions of the isolated aortic strip to adrenaline and noradrenaline, equally well before and after treatment with iproniazid. From this he concluded that the potentiating effect of cocaine was not due to inhibition of monoamine oxidase. It was decided to use a similar technique to determne whether the potentiation by imipramine could be due to inhibition of o-methyl transferase. Pyrogallol is known to interfere with the activity of o-methyl transferase (Axelrod & Laroche, 1959). Therefore, experiments were carried out to see whether imipramine potentiated the response of the decentralized nictitating membrane to adrenaline and noradrenaline equally well after pretreatment with pyrogallol as in the absence of pyrogallol.

Three experiments were carried out in which pyrogallol was given shortly before imipramine. Pyrogallol alone had a slight potentiating effect on the contractions to adrenaline but not to noradrenaline ; the height of the contractions to adrenaline was increased from an average of 4 mm before to 14 mm after 3 to 10 mg/kg of pyrogallol, and higher doses (20 to 30 mg/kg) produced no further potentiation. When imipramine (1 mg/kg) was injected after pyrogallol (20 to 30 mg/kg), a much greater potentiation of the responses to adrenaline and noradrenaline was obtained: the contraction to adrenaline was increased from an average of 8 mm before to 62 mm after imipramine, and the contraction to noradrenaline was increased from 3 mm before to 52 mm after imipramine. This effect was certainly not less than that obtained with imipramine alone (Fig. 1). Thus it is concluded that the potentiating effect of imipramine is not due to inhibition of o-methyl transferase.

Pipradol. Pipradol (2 mg/kg) had virtually no effect on the tone of the acutely decentralized membrane (out of 10 experiments a small contraction of 10 mm was observed in only one experiment). Like cocaine and imipramine, pipradol potentiated the contractions to adrenaline and noradrenaline, but not to tyramine (Table 1, Figs. 1 and 5), and the potentiation was maximal in 10 min. Pipradol also potentiated the effects of submaximal stimulation of the preganglionic and postganglionic nerve trunks (Figs. 1 and 5). There was a further similarity to cocaine, since pipradol (2 mg/kg) caused an immediate relaxation of the decentralized membrane made to contract by dexamphetamine (2 experiments).

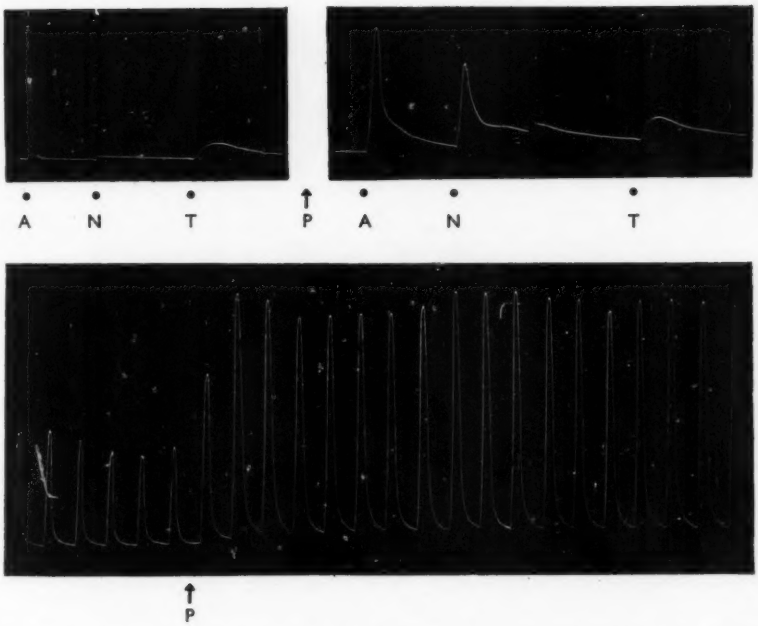


Fig. 5. Spinal cats. Records of contractions of acutely decentralized nictitating membranes in two experiments. Above: A=adrenaline, 4 μg; N=noradrenaline, 20 μg; and T=tyramine, 1 mg. Below: submaximal stimulation of preganglionic cervical sympathetic nerve. P=pipradol, 2 mg/kg. Time scale=1 min. All drugs were injected intravenously.

Nialamide. Nialamide, in a dose of 15 mg/kg, had no effect on the tone of the acutely decentralized membrane (4 experiments) when observations were continued for 2 to 4 hr after the injection. There was no obvious effect on the contractions produced by adrenaline or noradrenaline, but the size and duration of the contractions to tyramine were potentiated (Table 1, Figs. 1 and 6). The potentiation of the response to tyramine was first observed 1.5 hr to 4 hr after the injection of nialamide. Whereas control injections of up to 4 mg of tyramine produced a maximum duration of contraction of 5 min in different experiments, after nialamide the contraction due to tyramine persisted for periods exceeding 30 to 60 min: observations were not made over longer periods. Because of this greatly prolonged action of tyramine it was not possible to determine when nialamide had produced a maximum effect. Since the action of nialamide was slow in onset it was not practicable to test its effects on submaximal nerve stimulation.

Pheniprazine. Unlike the four compounds described previously, pheniprazine produced a marked contraction of the acutely decentralized membrane (Table 1). A dose of 2 mg/kg (two experiments) produced contractions of 45 mm and 120 mm respectively, with durations of more than 20 min. A dose of 1 mg/kg (10 experiments) produced contractions of 6 to 75 mm (average 29 ± 10 mm) and the duration

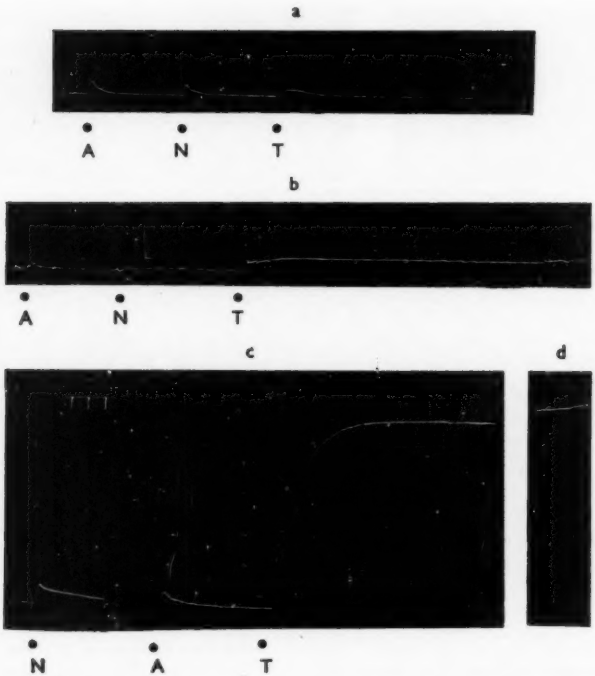


Fig. 6. Spinal cat. Records of contractions of acutely decentralized nictitating membrane. A= adrenaline, 4 μg; N=noradrenaline, 20 μg; T=tyramine, 1 mg. (*a*) Control; (*b*) 1 hr after 15 mg/kg of nialamide; (*c*) 2 hr later; (*d*) 20 min after (*c*). Time scale=1 min. All drugs were injected intravenously.

varied from 4 to 8 min. However, in 3 experiments, after 0.5 to 1 hr, there was a second more gradual increase in the tone of the membrane. An injection of 0.5 mg/kg caused only a small contraction (average 1 ± 0.8 mm in 5 experiments). Therefore, doses of 0.5 to 1 mg/kg, which usually produced relatively brief effects, were used in most of the experiments. These contractions could not have been due to nicotine-like ganglion stimulant effects such as Reinert (1959) has described for amphetamine, since in two experiments contractions were still observed with pheniprazine after removal of the superior cervical ganglion (Fig. 8).

Pheniprazine potentiated the effect of adrenaline and noradrenaline about 3 times, and the response to tyramine was potentiated more than 5 times (Table 1, Figs. 1 and 7). Although there was only a slight increase in the duration of the contractions to adrenaline and noradrenaline, the response to tyramine was considerably prolonged (Table 1 and Fig. 7).

Potentiation of the contractions to the three sympathomimetic amines was observed within 15 min of the injection of pheniprazine, but the maximum potentiation of the contractions to adrenaline and noradrenaline was not observed

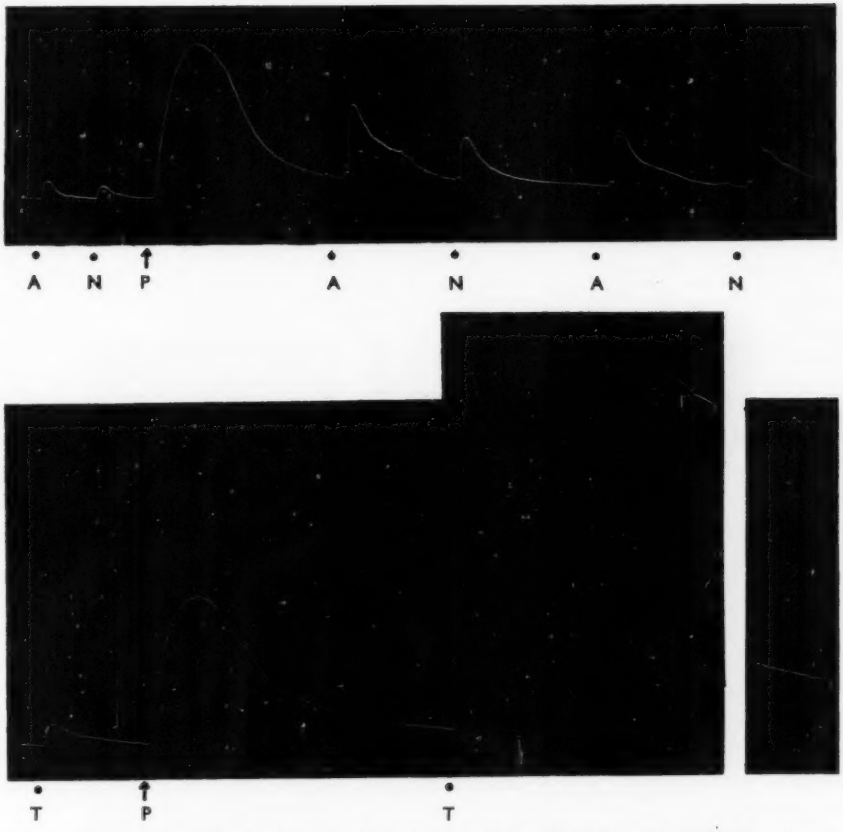


Fig. 7. Spinal cats. Records of contractions of acutely decentralized nictitating membrane in two experiments. P=pheniprazine, 1 mg/kg. Above: A=adrenaline, 5 μg; N=noradrenaline, 20 μg. Below: T=tyramine, 1 mg; interval of 20 min between records. Time scale=1 min. All drugs were injected intravenously.

until 0.5 to 3 hr after pheniprazine. No attempt was made to determine when a maximum effect on the tyramine response had been achieved. Since 5-hydroxytryptamine is a good substrate for monoamine oxidase (Blaschko, 1957), a few experiments were carried out to investigate the potentiating effect of pheniprazine on 5-hydroxytryptamine. The average contraction to 20 to 40 μg of 5-hydroxytryptamine was 5 mm. After 1 mg/kg of pheniprazine the average contraction to 5-hydroxytryptamine was 20 mm and the maximum potentiation was observed in 30 to 150 min (3 experiments). This effect was slight in comparison with the potentiation of the contractions to tyramine (Figs. 1 and 7). This may be due to the fact that there may be other metabolic pathways for 5-hydroxytryptamine, other than monoamine oxidase, which are not important to tyramine. For example,

Weissbach, Redfield & Udenfriend (1958) have shown that conversion to the O-glucuronide may be an important metabolic pathway for 5-hydroxytryptamine.

Fig. 8 illustrates one of the experiments summarized in Fig. 1, in which the effect of pheniprazine on submaximal sympathetic nerve stimulation was investigated. An injection of 0.5 mg/kg potentiated the contractions produced by stimulation of the preganglionic and postganglionic sympathetic nerves. A higher dose of 1 mg/kg caused both membranes to contract. Since the superior cervical ganglion on one side had been removed, neither the potentiation of the contractions to nerve stimulation nor the contraction produced by pheniprazine *per se* could be explained by a stimulant effect on the ganglion.

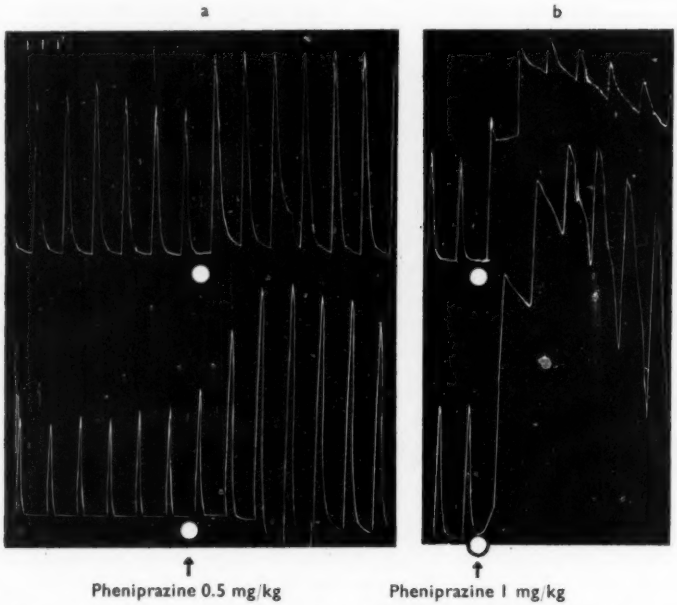


Fig. 8. Spinal cat. Records of contractions of nictitating membranes to submaximal sympathetic nerve stimulation. Above: acutely decentralized membrane (preganglionic stimulation). Below: acutely denervated membrane (postganglionic stimulation). Time scale=1 min. At the white dot pheniprazine was injected intravenously.

Dexamphetamine. Like pheniprazine, dexamphetamine caused a contraction of both the decentralized membrane (Table 1) and the acutely denervated membrane. The duration of these contractions was prolonged (greater than 30 min) at all of the doses used.

Since Reinert (1959) showed that amphetamine had a nicotine-like stimulant effect on the superior cervical ganglion when the drug was injected into the fluid perfusing the ganglion, it was of interest to determine whether the contraction of the decentralized membrane produced by an intravenous injection of amphetamine was

partly due to an effect on the ganglion. Thus in 3 experiments hexamethonium (5 to 10 mg/kg) was injected during the contraction produced by an intravenous injection of dexamphetamine (0.2 to 1 mg/kg). Since there was no effect whatever on the contraction, it is concluded that on intravenous injection the contraction produced by dexamphetamine was not due to a stimulant effect on the ganglion.

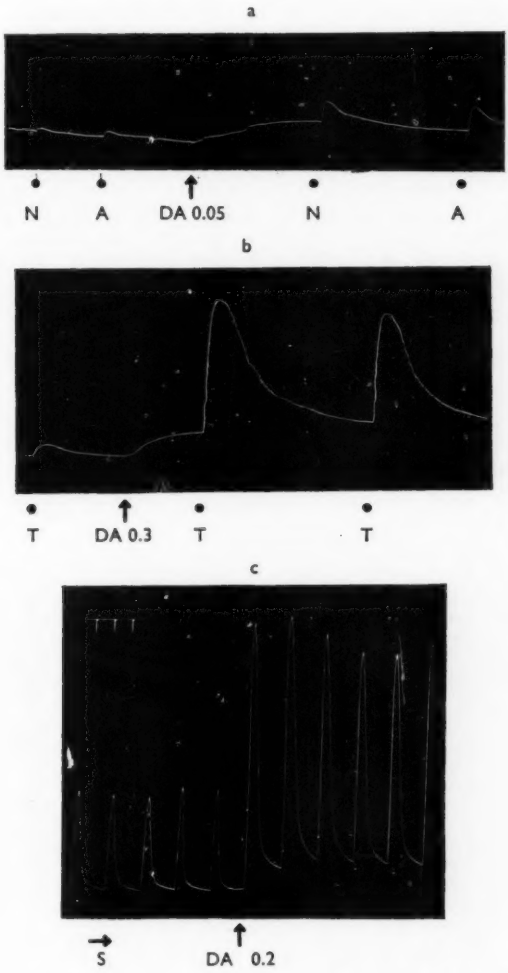


Fig. 9. Spinal cats. Records of contractions of acutely decentralized nictitating membrane in three experiments (*a*, *b* and *c*). DA 0.05, DA 0.2 and DA 0.3 = dexamphetamine 0.05, 0.2 and 0.3 mg/kg respectively. N = noradrenaline, 5 μg; A = adrenaline, 1 μg; T = tyramine, 1 mg; and S = submaximal stimulation of preganglionic cervical sympathetic nerve. Time scale = 1 min. All drugs were injected intravenously.

This is in agreement with observations made by Bejrablaya, Burn & Walker (1958), who found that hexamethonium did not reduce the stimulant effect of amphetamine on the heart.

Dexamphetamine 0.05 to 0.3 mg/kg slightly increased the size of the contractions produced by adrenaline and noradrenaline, but had a more marked effect on the response to tyramine (Table 1, Figs. 1 and 9). A maximum potentiation was observed 30 to 60 min after the injection of dexamphetamine. However, this apparent potentiation may be partly due to an additive action (Gaddum, 1948), since dexamphetamine itself caused prolonged contractions at these doses. Although the size of the contractions produced by tyramine was potentiated, there was no potentiation of the duration of the contractions (Table 1). In this respect dexamphetamine differed from pheniprazine.

Dexamphetamine produced extremely variable effects on the contractions produced by submaximal stimulation of the preganglionic and the postganglionic nerve. The effect on preganglionic stimulation varied from no effect in one experiment to the large effect shown in Fig. 9, where dexamphetamine increased the size of the contractions from 28 mm control to 76 mm. A similar effect was observed in one other experiment, but in 3 experiments there was only a 25% increase in the height of the contraction. In these last 3 experiments the effect of dexamphetamine on stimulation of the postganglionic nerve trunk was also determined: there was only a small potentiation, similar to that observed on preganglionic stimulation (Fig. 1).

Effects on the blood pressure. The major part of the investigation was concerned with effects on the nictitating membrane. However, since the blood pressure was

TABLE 3

EFFECT OF COCAINE AND ANTIDEPRESSANTS ON PRESSOR RESPONSES TO ADRENALINE, NORADRENALINE AND TYRAMINE IN THE SPINAL CAT

Doses of adrenaline and noradrenaline ranged from 1 to 40 μg, those of tyramine from 0·25 to 2 mg. * No significant result could be obtained since the effect of nialamide was very slow in onset, and increases in pressor responses to adrenaline and noradrenaline may have been due to spontaneous changes in sensitivity

	Intravenous dose mg/kg	No. of expts. showing at least 20 mm mercury increase (+) or decrease (−) in pressor response/total no. of expts.		
		Adrenaline	Noradrenaline	Tyramine
Cocaine	0·5–3·0	+5/5	+4/5	−5/5
Imipramine	1·0–4·0	+0/9	+5/8	−5/5
Pipradol	2·0	+3/4	+4/4	−3/4
Nialamide	15·0	No result*	No result*	+4/4
Pheniprazine	1·0	+3/4	+3/4	+3/4
Dexamphetamine	0·05–0·3	+3/5	+3/5	−1/3

recorded simultaneously, it is of interest to describe briefly the effects of cocaine and the antidepressant drugs on the pressor responses to adrenaline, noradrenaline and tyramine, and Table 3 summarizes the results obtained.

On the whole the results were similar to those obtained on the nictitating membrane, but there were a few differences. Firstly, imipramine potentiated the pressor response

to noradrenaline, but not the response to adrenaline, whereas on the nictitating membrane the contractions to both catecholamines were potentiated. Courvoisier, Leau, Ducrot, Fournel, Julou & Bardone (1959) and Sigg (1959) also found that imipramine potentiated the pressor effect of noradrenaline but not that of adrenaline. Secondly, pipradol reduced the pressor effect of tyramine, whereas it had no effect on the contraction of the nictitating membrane to tyramine, and, thirdly, dexamphetamine did not potentiate the pressor effect of tyramine, in contrast to the result obtained on the nictitating membrane, when the height (but not the duration) of the contractions to tyramine was potentiated.

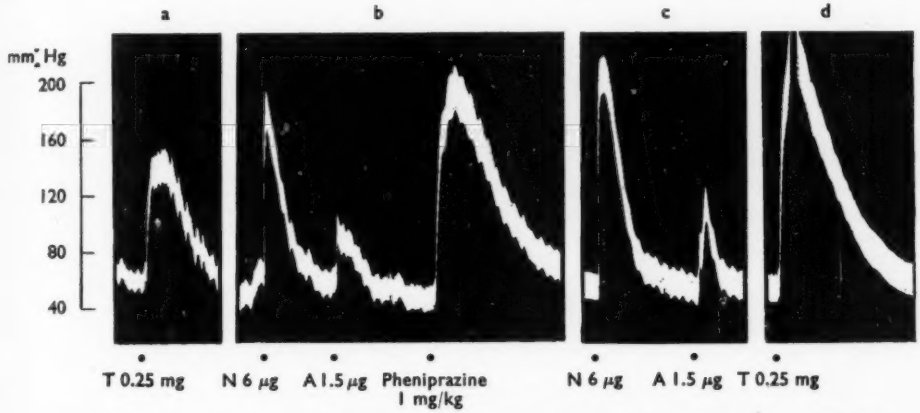


Fig. 10. Spinal cat. Effect of pheniprazine on pressor responses to tyramine (T), noradrenaline (N), and adrenaline (A). Interval of 20 min between *a* and *b*, 8 min between *b* and *c*, and 8 min between *c* and *d*. Time scale=1 min. All drugs were injected intravenously.

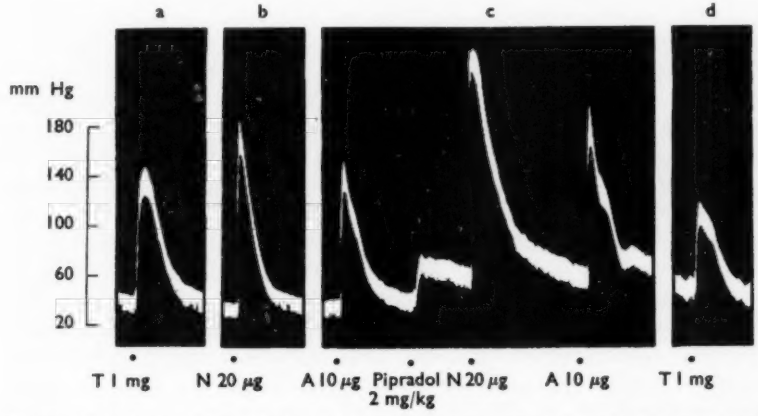


Fig. 11. Spinal cat. Effect of pipradol on pressor responses to tyramine (T), noradrenaline (N), and adrenaline (A). Interval of 10 min between *a* and *b*, 10 min between *b* and *c*, and 15 min between *c* and *d*. Time scale=1 min. All drugs were injected intravenously.

All the compounds, with the exception of nialamide, produced pressor effects. The average pressor effect obtained with cocaine, imipramine and pipradol was of the order of 25 mm of mercury, whereas after pheniprazine or dexamphetamine the average pressor responses were 85 and 50 mm of mercury respectively.

Figs. 10 and 11 illustrate experiments with pheniprazine and pipradol. Pheniprazine (Fig. 10) potentiated the pressor effects of adrenaline, noradrenaline and tyramine, whereas pipradol (Fig. 11) potentiated the action of adrenaline and noradrenaline, but reduced the effect of tyramine.

DISCUSSION

The effects of cocaine were very interesting. It contracted the innervated nictitating membrane, which is in agreement with the earler observation of Burn & Robinson (1952), and, although the size and duration of the contractions varied considerably, in those experiments in which they were sufficiently prolonged the membrane relaxed immediately when either the preganglionic nerve was cut or when hexamethonium was injected. Adrenaline or noradrenaline also caused a relaxation of the membrane, and this effect is thought to be due to the ganglion blocking action of these amines (Marrazzi, 1939 ; Bülbring, 1944 ; and Matthews, 1956).

One tentative explanation of the above action of cocaine is that it might increase the nervous activity in the preganglionic nerve, but this has not been investigated. Macmillan (1959) suggested that cocaine potentiated the action of catecholamines by reducing their uptake by storage sites and thus allowing more to occupy receptor sites. This hypothesis is in agreement with the results of Whitby, Hertting & Axelrod (1960), who found that cocaine reduced the uptake of injected noradrenaline by the tissues and raised the plasma concentrations. Therefore, an alternative explanation of the contraction to cocaine is that cocaine reduces the uptake by storage sites of the noradrenaline released by spontaneous activity in the sympathetic nerve. It is unlikely that cocaine actually increases the amount of noradrenaline released by the spontaneous activity in the sympathetic nerve fibres, since Trendelenburg (1959) could not find any increase in the output of noradrenaline from the spleen on stimulation of the splenic nerve after cocaine.

The effects of cocaine and the antidepressant drugs on the nictitating membrane could be divided into three main types. The first type of effect was a potentiation of the contractions to adrenaline or noradrenaline, accompanied by no effect on, or a reduction of the contractions to, tyramine. Cocaine, imipramine and pipradol had this type of effect. Furthermore, Fleckenstein & Stöckle (1955) found that cocaine antagonized the contractions to amphetamine, and it has now been shown that imipramine and pipradol have a similar action to cocaine in antagonizing the contractions to amphetamine. Recently Axelrod, Whitby & Hertting (1961) have demonstrated a further similarity between cocaine and imipramine: both reduced the uptake of noradrenaline by the tissues. Therefore, it is possible that cocaine, imipramine and pipradol potentiate the effects of adrenaline and noradrenaline by the same mechanism.

The second type of effect observed was a potentiation of the height and duration of the contractions to tyramine, as shown by pheniprazine and nialamide. Since

tyramine is a good substrate for monoamine oxidase (Wiener, 1960), and both pheniprazine and nialamide are potent inhibitors of this enzyme (Zbinden, Randall & Moe, 1960), it is reasonable to conclude that the potentiation of the response to tyramine by pheniprazine and nialamide is due to inhibition of monoamine oxidase. Dexamphetamine also increased the size of the contractions to tyramine. However, dexamphetamine did not prolong the contractions to tyramine or potentiate its pressor effect, and dexamphetamine is only a weak inhibitor of monamine oxidase (Zbinden *et al.*, 1960). Therefore, it is unlikely that dexamphetamine potentiated the size of the contraction to tyramine by an inhibitory eflect on monoamine oxidase.

The significance of monoamine oxidase in limiting the action of catecholamines is not clear. However, Spector, Kuntzman, Shore & Brodie (1960) have suggested that, whereas catechol-O-methyltransferase may be important in the inactivation of circulating catecholamines, it is monoamine oxidase which is important at the site of formation and release of these amines in tissues such as the brain and the heart.

The third type of effect was a direct stimulant action on the membrane. This effect was shown by dexamphetamine and pheniprazine. Since Reinert (1959) found that amphetamine had a nicotine-like action on the superior cervical ganglion when the drug was injected into the fluid perfusing the ganglion, it was noteworthy that intravenous injections of dexamphetamine or pheniprazine caused contractions of the membrane after removal of the ganglion. Therefore, the contractions obtained on intravenous injection were clearly not due to nicotine-like ganglion stimulant effects.

Trendelenburg (1959) found that cocaine potentiated the contractions of the membrane to submaximal stimulation of the preganglionic and postganglionic sympathetic nerves. Thus, it was extremely interesting to find that imipramine, pipradol, pheniprazine and dexamphetamine, all of which have central stimulant or antidepressant properties, had similar effects to cocaine. Furthermore, Kamijo, Koelle & Wagner (1956) found that another antidepressant drug, iproniazid, also potentiated the contractions to preganglionic nerve stimulation.

It is possible that cocaine, imipramine and dexamphetamine potentiate sympathetic nerve stimulation by the same mechanism since they reduce the uptake of noradrenaline by the tissues (Whitby *et al.*, 1960 ; Axelrod *et al.*, 1961), but it is not known whether pipradol has the same effect. Axelrod *et al.* (1961) also showed that pheniprazine did not affect the tissue levels of noradrenaline and therefore there must be an alternative explanation (possibly inhibition of monoamine oxidase) for pheniprazine.

Since it seems likely that a central adrenergic mechanism may be important in the maintenance of alert behaviour (Dell, Bonvallet & Hugelin, 1954 ; Rothballer, 1959), it is tempting to speculate that there may be a similarity between the effects of these central stimulant and antidepressant drugs on electrical stimulation of peripheral adrenergic nerves and their effects on catecholamines released within the brain. For two of these drugs, cocaine and amphetamine, there is some suggestive evidence (Rothballer, 1957) for potentiation of the central effects of adrenaline ; they were shown to increase the alerting effect of injected adrenaline as shown on the electroencephalogram.

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STUDIES ON A NEW INTRAMUSCULAR HAEMATINIC, IRON-SORBITOL

BY

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A new iron preparation for intramuscular use is described. It contains a complex of iron, sorbitol and citric acid. Its properties in comparison with several other complexes, particularly iron-dextran, have been studied. The preparation is stable in serum, is hypertonic, does not produce haemolysis, and affects coagulation only at very high concentrations, such as are reached only *in vitro*. Absorption from muscle takes place very rapidly; two-thirds of the iron is removed within 3 hr, and there is a very rapid increase in the serum-iron concentration. In experimental animals, the maximum level is reached after about 20 min and in man after about 2 hr. Disappearance from the serum takes place rapidly. The preparation contains a small amount of a fraction which reacts with transferrin and is dialysable. In man, about 30% of the total dose of iron is excreted through the kidneys during the first 24 hr after injection, the greater part of the excretion taking place during the first few hours.

Parenteral iron therapy has been increasingly used since Nissim (1947) discovered that saccharated oxide of iron could be used for intravenous administration. This preparation produces severe local side-reactions and cannot be administered intramuscularly (Slack, 1949). Agner, Andersson & Nordenson (1948) studied an iron preparation for intravenous use in which the iron was present as a special ferri-dextrin complex in colloidal form. Later investigations by Andersson & Bergström (1956) demonstrated that this iron compound could also be administered intramuscularly in man.

Complex compounds of the iron-dextran type (Fletcher & London, 1954), ferric disodium-N-hydroxyethyl-ethylene-diamine-triacetate (Seven & Peterson, 1958) and ferric choline citrate (Virtanen & Hartiala, 1958) have also been investigated, and of these the iron-dextran complex is used clinically (Baird & Podmore, 1954; Cappell, Hutchison, Hendry & Conway, 1954; Scott & Govan, 1954; Jennison & Ellis, 1954). This compound is said to be a low molecular dextran-iron complex and has a low toxicity. Its pharmacology has been studied by Martin, Bates, Beresford, Donaldson, McDonald, Dunlop, Sheard, London & Twigg (1955). Beresford, Golberg & Smith (1957) investigated the absorption mechanism and the local effect of the compound on muscle, and Nordén (1957), Grimes & Hutt (1957) and Karlefors & Nordén (1958) studied in man the metabolism of an intramuscularly administered iron-dextran complex labelled with ^{59}Fe.

According to these more recent investigations 50% to 80% of the dose of iron given was absorbed from the site of injection within 20 to 100 days while the residual iron remained in the muscle for a long period. A search has therefore been made for an iron preparation for intramuscular injection which can be absorbed more rapidly and completely.

Sorbitol is known to improve the absorption of oral ferrous sulphate (Herndon, Rice, Tucker, van Loon & Greenberg, 1958). The possibility of producing new iron complexes for intramuscular injection, containing sorbitol or even other substances, has been studied by Lindvall & Högberg (unpublished observation). The resulting preparations have been tested, and the product with the best properties is described here.

METHODS

Characterization of the preparations used

This is a complex of iron, sorbitol and citric acid, containing, in addition, dextrin. It is prepared by adding an aqueous solution of ferric chloride in portions to a solution of 60° C temperature containing sorbitol, citric acid and dextrin. The *p*H of the solution is adjusted to weakly alkaline after the addition of each portion. After cooling, the complex is precipitated by adding alcohol to the mixture. The iron content of the complex is 14±2%. A solution of it is sterilized by autoclaving and contains 50±2 mg/ml. elemental iron and has a *p*H of 7.5±0.2 [Jectofer, Astra]. This solution is referred to as iron-sorbitol.

Electrophoretic investigations with a paper-strip electrophoresis apparatus from L.K.B. Produkter Fabriks A.B., Sweden, in a 0.1 M phosphate buffer at *p*H 7.6 show that the compound contains at least two iron-containing components which migrate towards the anode (Fig. 1). Furthermore, about 6% of the iron is found in a more rapidly moving form in investigations with a Spinco continuous-flow paper electrophoresis using 0.07 N acetate buffer of *p*H 5.0 (Fig. 2). This part of the compound is of lower molecular weight and dialysable. Moreover, it will be seen from Fig. 2 that the dextrin can be separated from the iron-containing fractions. It is presumed that dextrin acts as a stabilizer (Eriksson, unpublished observation).

Ultracentrifugation of the poly-dispersed iron compound gives an upper limit for the sedimentation constant of 8 to 9 Swedberg units. As the density of the molecule is unknown, it is not possible to calculate the exact molecular weight. With certain assumptions, the probable average molecular weight is estimated to be below 5,000 (Eriksson, 1962).

A solution of iron-dextran containing Fe 50 mg/ml. and with a *p*H of 5.8 [Imferon, Benger] was used as a basis of comparison. In some of these experiments, solutions of the iron-dextrin complex containing Fe 20 mg/ml. and with a *p*H of 7.4. [Astrafer, Ferrigen, Astra], as well as solutions of saccharated oxide of iron containing Fe 20 mg/ml. and with a *p*H of 10.9 [Intrafer, Pharmacia], were also used.

Stability at different p*H*

The *p*H of the preparations in aqueous solutions was regulated within the range 1 to 8, with 0.1 and 1.0 N hydrochloric acid, in accordance with the method of Nissim & Robson (1949). After the addition of the hydrochloric acid the iron concentration in all the solutions was Fe 1 mg/ml. After the solutions had been standing for 24 hr at room temperature the precipitate was removed by centrifugation and the iron content and *p*H in the supernatant determined. The iron was estimated colorimetrically by means of ammonium thiocyanate.

Haemolytic effect

This was studied by mixing 1.0 ml. of solutions of the iron complexes containing Fe 0.04 to 20.0 mg/ml. of 0.9% sodium chloride solution with 0.43 ml. of blood corpuscle suspension consisting of 1.0 ml. of rabbit blood in 25 ml. of physiological saline. After the mixture

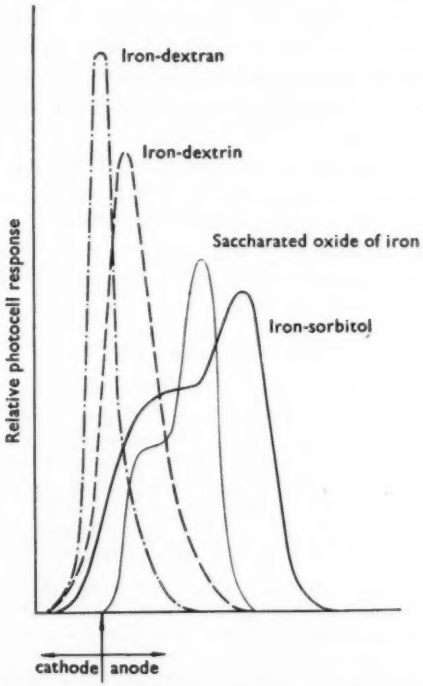

Fig. 1. Paper electrophoresis of iron-sorbitol, iron-dextran, iron-dextrin and saccharated oxide of iron in phosphate buffer at *p*H 7.6. The fractions are stained for iron by using potassium ferrocyanide in 1 N hydrochloric acid.

had been standing for 3 hr at room temperature the non-haemolysed corpuscles were removed by centrifugation. After having been washed twice with 3.5 ml. of physiological saline the blood cells were haemolysed with 5 ml. of 0.05% ammonium hydroxide solution (5 ml./100 ml. of concentrated ammonia). The extinction was read at 576 mμ.

Anticoagulant activity

This was measured *in vitro* by the method of Nissim (1954). 1 ml. of blood was taken from the carotid artery of a rabbit and transferred directly to tubes containing 0.32 ml. of iron solutions with an iron content of 0.045 to 25 mg/ml. Fe of physiological saline. After thorough mixing, the time at which coagulation occurred was measured.

Intramuscular absorption of iron from site of injection

The iron preparations were injected deep into the glutei of rabbits. (Male albino rabbits weighing 2 to 3 kg were used consistently.) The animals were killed at different time intervals after injection and the gluteal muscles were dissected away from the leg. The muscles and skin at the site of injection were wet-oxidized with sulphuric and nitric acid and the iron determined colorimetrically by means of ammonium thiocyanate. The residual iron was obtained by subtracting the iron content of normal muscle.

Iron concentration in serum

The serum-iron was estimated according to the principles of Heilmeyer & Plötner (1937). 1.0 ml. of serum was mixed with 1.5 ml. of 4 N hydrochloric acid and hydrolysed at 50° C

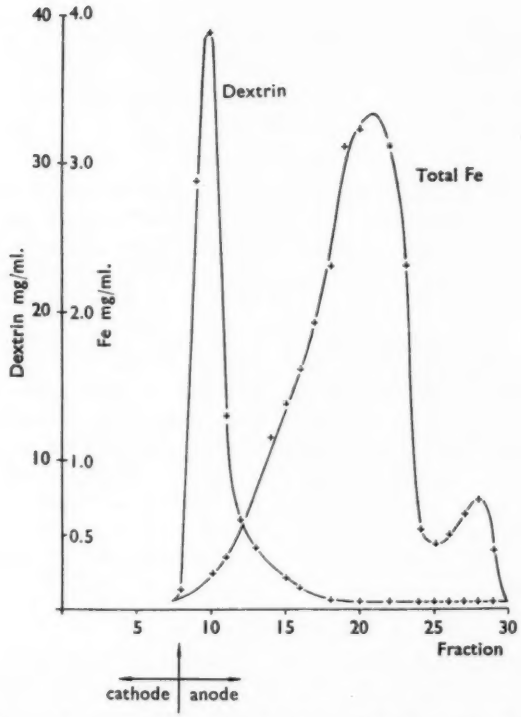

Fig. 2. Continuous-flow paper electrophoresis of iron-sorbitol in acetate buffer at *p*H 5.0.

for 20 min. After hydrolysis, the protein was precipitated with 1.5 ml. of 20% trichloroacetic acid solution. Ten minutes later, the precipitate was centrifuged off at 3,500 g for 30 min. 3.0 ml. of the clear supernatant was transferred to a 5.0 ml. volumetric flask; 5 drops of freshly prepared 0.2% ascorbic acid solution, 4 drops of 1% *ortho*-phenanthrolene in a 10% alcohol solution and 1 drop of a 0.2% 2,5-dinitrophenol solution in absolute alcohol were added with shaking. The sample was then neutralized with concentrated ammonia till the indicator turned yellow. The mixture was acidified with 0.4 N hydrochloric acid till the exact moment when the yellow colour disappeared, and the volume was adjusted to make 5.0 ml. The extinction was read in a Zeiss photometer at 515 mμ. The analytical values obtained by this method include plasma-bound iron and circulating iron preparations.

Diffusion of iron into tissue fluids

The diffusion into tissue fluids was studied by the method of Nissim (1953) with the peritoneum of the mouse as the dialysing membrane.

The mice were injected intraperitoneally with 2.0 ml. of physiological saline and immediately afterwards received iron-sorbitol and iron-dextran solutions by intravenous injection. The animals were killed with ether after different intervals, the peritoneal cavity was opened and the fluid in the abdominal cavity collected. Abdominal contents contaminated with blood were not used. The concentration of iron was determined with *ortho*-phenanthrolene in 3 ml. mixed samples from six mice according to the serum-iron method.

Iron in urine

The iron concentration was determined after the urine had been wet-oxidized with sulphuric acid and hydrogen peroxide in accordance with the *ortho*-phenanthrolene method.

RESULTS

Precipitation test

The solution of iron-sorbitol can be diluted with 0.9% sodium chloride solution to concentrations between 0.001 and Fe 25 mg/ml. without giving any visual precipitation. Nor does any precipitation take place after dilution with rabbit serum and 1% bovine fibrinogen solution to the same concentrations as above, when the solution is kept at 37° C for 2 hr.

The results from the studies about the stability of the iron preparations at different *p*H showed that iron-sorbitol precipitated within the *p*H range of 1.7 to 3.5, with maximum precipitation (40%) at *p*H 2.5. Between 3.5 and 8 there was no precipitation. Iron-dextran was stable between a *p*H of 1 and 8. Iron-dextrin showed some precipitation within the 1.0 to 2.3 range immediately after the addition of hydrochloric acid, but this disappeared after a few hours. No precipitation was observed at *p*H values exceeding 2.3. The saccharated oxide of iron used in this experiment diverged from that of Nissim. It was precipitated practically quantitatively at *p*H 4.5 to 7.0, and at *p*H 7.2 it showed 50% precipitation. At *p*H 7.6 no precipitation could be observed.

Osmotic properties

These were studied after dilution with distilled water to different concentrations. The depression of freezing point was determined with a Beckmann thermometer, and it was found that a solution, isotonic with blood, was obtained when the iron content in the diluted iron-sorbitol solution was Fe 12 mg/ml. The corresponding value for the iron-dextran preparation was Fe 35 mg/ml. The result shows that the preparations are hypertonic, especially iron-sorbitol.

Haemolytic effect

The results showed that iron-sorbitol and iron-dextrin had no haemolytic effect, while iron-dextran was haemolytic at a concentration above Fe 1.75 mg/ml. and saccharated oxide of iron had an effect at a concentration above Fe 0.44 mg/ml.

Anticoagulant activity

As may be seen from Table 1, iron-dextran as well as sorbitol alone had little effect on the coagulation. Iron-sorbitol and iron-dextrin, however, prolonged the clotting time in concentrations higher than 0.2 mg/ml. Fe ; at the highest concentrations, coagulation was completely inhibited.

The effect of iron-sorbitol on coagulation was studied *in vivo* in 8 rabbits after intravenous and intramuscular doses corresponding to Fe 1.5 and 5.0 mg/kg. No effect on the coagulation was observed after 2 to 60 min ; nor was any change in the clotting time noted in man, at the same times, after injection of doses corresponding to Fe 1.5 mg/kg in 3 healthy subjects.

TABLE 1

ANTICOAGULANT ACTIVITY OF THREE IRON PREPARATIONS AND SORBITOL *IN VITRO*

Concentration of Fe or sorbitol in mg/ml.	Clotting time in min			
	Iron-sorbitol	Iron-dextran	Iron-dextrin	Sorbitol
6·0	—	17	—	7
3·0	—	15	—	7
1·5	42	12	82	7
0·75	19	12	46	7
0·37	13	12	24	5
0·18	11	7	17	5
0·09	10	7	15	5
0·045	8	7	12	5
0·022	5	7	10	5
0·011	5	7	10	5

Absorption of iron after intramuscular injection

Iron-sorbitol and iron-dextran solutions were injected into 50 rabbits in a dose of Fe 20 mg/kg (0.4 ml.). The results showed that iron-sorbitol, when administered intramuscularly, was absorbed very rapidly ; after 3 hr about two-thirds of the iron had already been removed from the site of injection (Fig. 3). The corresponding

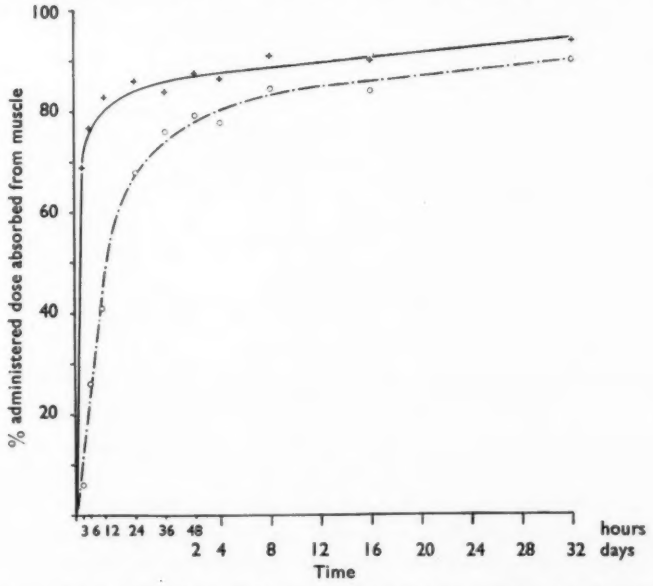

Fig. 3. Absorption of iron-sorbitol and iron-dextran after intramuscular injection in rabbit of a dose corresponding to Fe 20 mg/kg. ——— iron-sorbitol. — · — · — iron-dextran.

figure for iron-dextran after the same interval was only about 10%. For iron-sorbitol a rapid absorption phase was concluded after 12 hr, when the animals had 15 to 20% of the injected iron left at the site of administration. For iron-dextran, this phase was not completed until after 48 hr, and about 20% of the iron was then

still present in the muscle. At dissection it was seen that the muscle was free from the iron preparation after these intervals but that the fatty tissue between the muscles and the subcutaneous fat was stained with iron. The residual iron subsequently disappeared at a very slow rate; 32 days after the injection about 6% of the iron was still present in the specimens from the animals which had received iron-sorbitol and 9% in those which had received iron-dextran.

Iron content in serum after intramuscular injection

The iron content in serum was estimated in 20 rabbits at different time intervals after intramuscular injection of solutions of iron-sorbitol and iron-dextran in a dose corresponding to 1.5 Fe mg/kg. Blood samples were taken 6 to 8 times from the marginal ear vein of each rabbit. The values are given in Figs. 4 and 5; each point is the mean of 5 estimations.

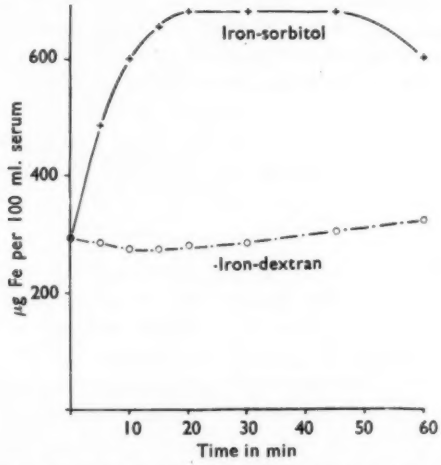

Fig. 4. Serum-iron during the first hour after intramuscular injection in rabbit of iron-sorbitol and iron-dextran in doses corresponding to Fe 1.5 mg/kg.

After iron-sorbitol there was a rapid rise in the serum-iron content; the maximum level was reached after about 20 min and was maintained for a further 30 min. With iron-dextran a definite increase was not recorded until about 3 hr after administration, and the maximum value was reached after 6 hr (Figs. 4 and 5). Similar tests were carried out with iron-sorbitol in man, doses corresponding to 25, 50 and 100 mg Fe being administered deep intramuscularly in the gluteal region in respectively 4, 4 and 5 healthy subjects. As may be seen from Fig. 6, the serum-iron began to increase immediately after the injection, reached the maximum about 2 hr later, and then decreased gradually.

Iron-binding capacity of serum

The unsaturated iron-binding capacity of serum was determined by the method of Cartwright & Wintrobe (1949) after both intravenous and intramuscular injection

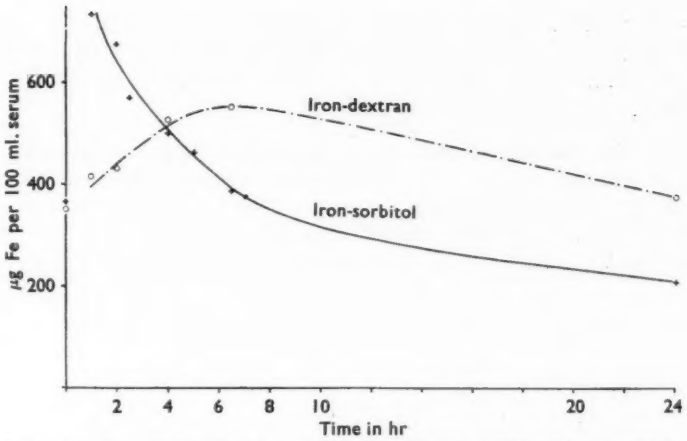

Fig. 5. Serum-iron 2 to 24 hr after intramuscular injection in rabbit of iron-sorbitol and iron-dextran in a dose corresponding to Fe 1.5 mg/kg.

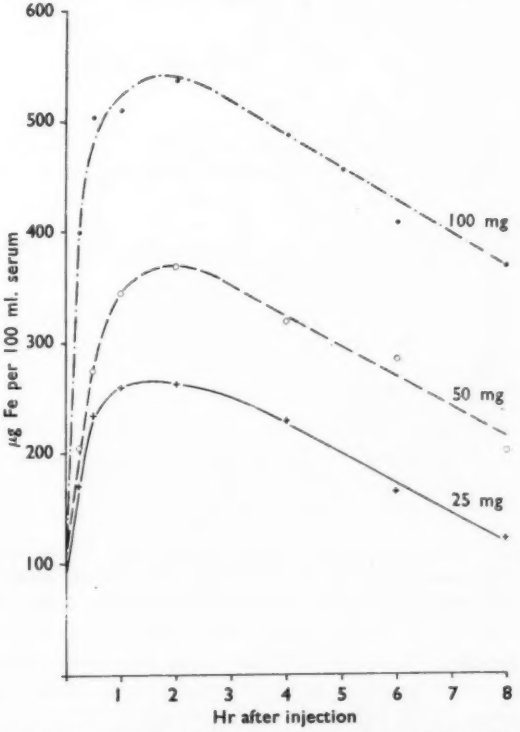

Fig. 6. Serum-iron after intramuscular injection in man of iron-sorbitol in doses corresponding to 25, 50 and 100 mg Fe.

of iron-sorbitol into rabbits in doses equivalent to Fe 1.5 mg/kg. In these preliminary experiments it was established that the transferrin was saturated with iron during the first 6 hr. After 24 hr the serum showed the same values for the unsaturated iron-binding capacity as before the injection.

The values for the unsaturated iron-binding capacity of the human subjects used for the determination of the iron content in serum were also examined. The values decreased following the injections. The decrease was most marked after the 100 mg dose, which produced complete saturation of the transferrin 0.5 to 6 hr after the injection (Fig. 7). The levels of the total iron-binding capacities of the respective

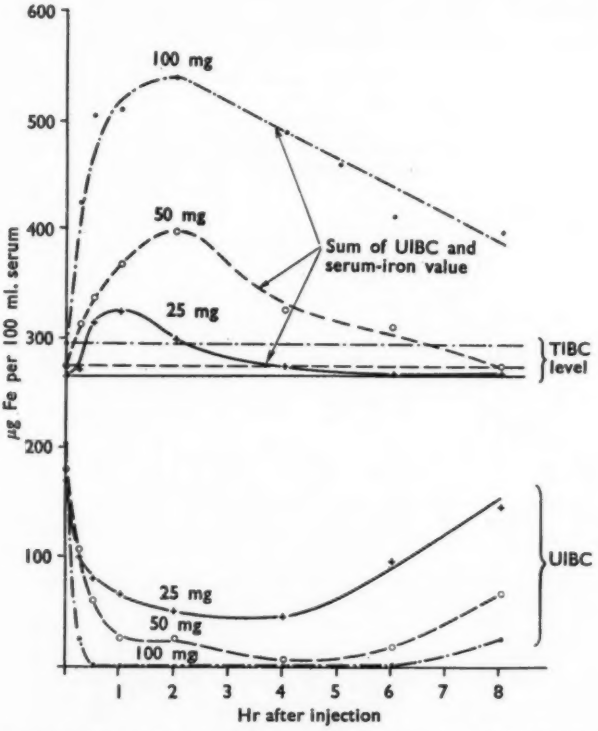

Fig. 7. Unsaturated iron-binding capacity (UIBC) and the sum of UIBC and serum-iron value after intramuscular injection in man of iron-sorbitol in doses corresponding to 25, 50 and 100 mg Fe. The levels for total iron-binding capacity (TIBC) are also shown.

groups are also indicated in the figure. These levels have been determined by adding the values of the unsaturated iron-binding capacity to the serum-iron values of the treated subjects prior to the injection of iron-sorbitol. The sum of the unsaturated iron-binding capacity and the serum-iron value has also been calculated at different times after the injection of iron-sorbitol.

During the first 6 hr this sum, as can be seen from Fig. 7, exceeds the total iron-binding capacity as found before the injection. The reason for this is that the value of the iron content of serum after the injection includes, as mentioned above, both iron bound to plasma and circulating iron-sorbitol constituents not directly bound to plasma proteins. An attempt to remove this surplus iron, by chromatography with alumina by the method of Laurell, showed that the surplus was not adsorbable as is the case with iron-dextran (Laurell, 1958).

In *in vitro* experiments, it was found that the iron-sorbitol solution could react with, and saturate, transferrin. Closer study established that this property was bound to the low-molecular and dialysable part of iron-sorbitol. The dialysate, however, needed a larger amount of iron in order to saturate the transferrin than a standard solution containing ferrous nitrate. The results are given in Fig. 8.

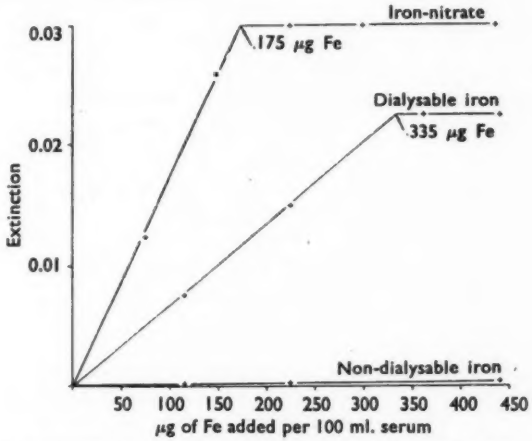

Fig. 8. Saturation of transferrin *in vitro* by adding iron-nitrate, dialysable and non-dialysable parts of iron-sorbitol to rabbit serum.

Iron content in serum after intravenous injection

The iron content of the serum was estimated at different intervals after intravenous injection of the iron preparations into rabbits in doses corresponding to 1.5 mg/kg. Iron-sorbitol, iron-dextran, iron-dextrin and saccharated oxide of iron were injected into the left ear of respectively 16, 8, 3 and 3 animals; blood specimens were taken from the right ear.

The results have been assembled in Fig. 9, which shows that, about 4 hr after the injection of iron-sorbitol, iron-dextrin and saccharated oxide of iron, the iron concentration in the serum was in the neighbourhood of the normal. Iron-dextran, however, was removed from the blood stream at a very slow rate.

Diffusion into tissue fluids

360 male albino mice weighing 20 g received iron preparations intravenously in doses corresponding to Fe 5, 10 and 25 mg/kg.

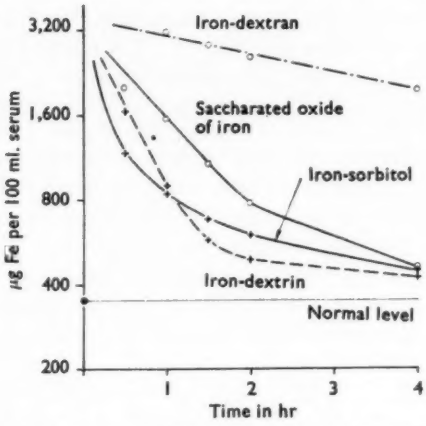

Fig. 9. Iron in serum after intravenous injection in rabbit of iron-sorbitol, iron-dextran, iron-dextrin and saccharated oxide of iron in a dose corresponding to Fe 1.5 mg/kg.

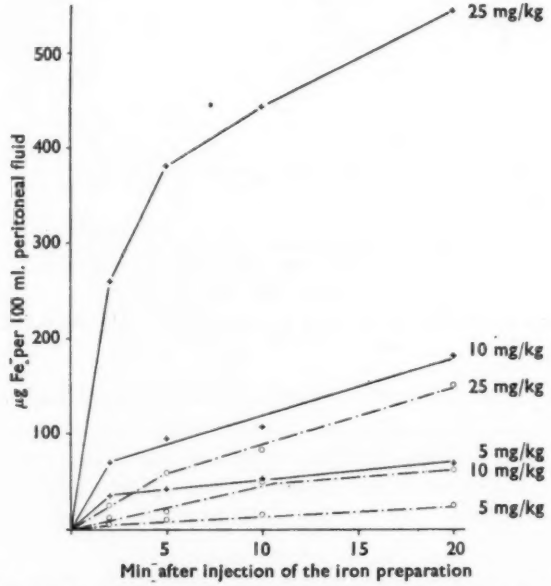

Fig. 10. Diffusion into the peritoneal cavity of iron-sorbitol and iron-dextran after intravenous injection in mice in doses corresponding to Fe 5, 10 and 25 mg/kg. ——— iron-sorbitol. —·—·— iron-dextran.

In Fig. 10 each point represents the mean of 3 determinations in the case of iron-sorbitol and 2 in the case of iron-dextran. It will be seen that both preparations diffused into the peritoneum in amounts to some extent proportional to the dose injected, though the diffusion of iron-dextran was much less.

Renal excretion

The renal excretion of iron-sorbitol following intramuscular injection into 4 rats (Sprague-Dawley) of doses corresponding to Fe 1.5 mg/kg was studied in preliminary experiments. The animals were placed in metabolism cages and the urine was collected during 24 hr. It was found that the rats excreted respectively 24.7, 26.1, 20.5 and 34.5% of the dose of iron injected within 24 hr.

More detailed studies were made of the urinary excretion pattern in man. Nine healthy subjects received 100 mg Fe by intramuscular injection and during the first 24 hr samples of urine were taken at different intervals after the injection. On an average, a total of 30% of the dose of iron given was excreted. As may be seen

TABLE 2

RENAL EXCRETION OF IRON AFTER INTRAMUSCULAR INJECTION IN MAN OF IRON-SORBITOL IN A DOSE EQUIVALENT TO 100 MG Fe

	Renal excretion as %				
	Hr after injection				
Patient	0–3	3–6	6–9	Over 9	Total
G. A., female	12·8	11·1	6·7	1·4	32·0
H. J., male	20·8	4·0	3·7	1·6	30·1
I. M., female	15·2	7·5	4·5	6·3	33·5
I. J., female	13·2	9·8	2·0	2·6	27·6
L. M., female	13·0	7·5	2·1	1·9	24·5
E. H., female	9·7	12·7	4·8	2·4	29·6
K. P., female	15·0	12·5	2·9	6·5	36·9
S. L., male	21·2	11·0	3·8	1·0	38·0
H. H., male	9·4	4·5	3·3	1·2	18·4
Mean value	14·5	9·0	3·8	2·8	30·1

from Table 2, the greater part of the excretion took place during the hours immediately following the injection, and in general it had been completed within 24 hr. The same percentage excretion was obtained after doses corresponding to 50 and 25 mg Fe.

DISCUSSION

According to Golberg (1958), absorption of the iron-dextran complex from the site of administration after an intramuscular injection takes place mainly during the first 72 hr. The absorption rate mentioned by Golberg for the iron-dextran complex has in the main been verified in the present investigation ; about 80% of the iron was absorbed within 48 hr of an injection of iron-dextran. For iron-sorbitol, the first absorption phase was very short ; two-thirds of the injected iron had already been cleared from the site of administration after 3 hr, and 12 hr after the injection an 80 to 85% absorption from the muscle was recorded. The rapid absorption of this complex is rendered possible partly because of its low mean molecular weight, which does not exceed 5,000, and also because, like iron-dextran, it is stable in tissue fluids and at a physiological *p*H.

The residual iron remaining at the site of injection after the first absorption phase is removed very slowly. Golberg (1958) observed that in the case of iron-dextran about 10% was still unabsorbed 15 days after administration into rabbits. In the present investigation, 9% of the iron was still present at the site of administration

32 days after injection of the iron-dextran complex. For iron-sorbitol about 6% of the iron remained uncleared at the end of the same period. A probable reason why some of the iron from both of the preparations was retained at the site of injection is that the iron complex may have spread to the subcutaneous fat and to the fatty tissues between the muscles where clearance is more difficult. The large dose of iron given to the experimental animals is also a factor which may have contributed towards producing a more extensive spread of the iron from muscle to fatty tissue.

The rapid absorption of iron-sorbitol from the site of injection was also apparent from the iron concentration in serum which, in the animal experiments, had already reached the maximum level after 20 min. Iron-dextran, on the other hand, produced no appreciable elevation of the iron content in serum during the first few hours. The difference between the behaviour of the two complexes in serum is due to the feature demonstrated by Svärd & Lindvall (1961), that iron-sorbitol is absorbed into the blood stream both directly and through lymphatic pathways, while iron-dextran is absorbed only via the lymphatic system.

When iron-sorbitol appears in the serum, after absorption, it has no effect on the clotting mechanism of the blood. *In vitro* experiments demonstrated, however, that iron-sorbitol as well as iron-dextrin had a certain inhibitory effect on coagulation. This inhibition, however, occurred only at concentrations considerably higher than those reached in clinical use. The citric acid content of the preparations is the cause of this *in vitro* inhibition.

Despite the rapid absorption that follows intramuscular injection of iron-sorbitol, the iron content of the serum was not as high as was expected, taking into consideration the blood volume and the amount of iron given. This is due to the fact that the absorbed iron disappears rapidly from the circulation, as was seen in the investigations on the clearance of intravenously administered iron preparations. Iron-sorbitol and iron-dextrin preparations disappeared from the blood stream at approximately the same rate while iron-dextran circulates for a long period. According to Andersson (1950), the uptake of iron-dextrin by the reticulo-endothelial system runs parallel to the disappearance from the blood. The iron-sorbitol is probably also taken up by these organs. The initial clearance of the iron-sorbitol preparation takes place more rapidly, however, than that of iron-dextrin, and this suggests that the elimination of the former is influenced by other factors as well. Filtration of the iron through glomeruli, which is made possible by the low molecular weight of iron-sorbitol, is a contributing factor in the rapid initial clearance. About 15% of the dose of iron injected in man was excreted into the urine within the first 3 hr of injection. Iron-sorbitol can, furthermore, diffuse into the tissue fluids.

Following intramuscular administration of the new preparation, and when the doses are large, a temporary saturation of the iron-binding capacity of the serum occurs both in man and in animals shortly after the injection. This property of the preparation is bound to the low-molecular, dialysable fraction. Owing to the fact that this fraction reacts with transferrin it is presumably immediately available for erythropoeisis while the other part of the preparation is taken up by the reticulo-endothelial system for further transformation and metabolism.

Our sincere thanks are due to Bertil Sjögren, M.D., Director of the Research Laboratories of Astra, for constructive criticism, and to all who have assisted in their various ways to make possible the publication of this paper.

Jectofer, Astra, is pending British patent application number 5791/61.

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ACTION OF BRETYLIUM AND GUANETHIDINE AT THE NEUROMUSCULAR JUNCTION

BY

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Bretylium and guanethidine produced a block of neuromuscular transmission in the rat phrenic nerve diaphragm and cat sciatic gastrocnemius preparations, but had a potentiating effect on acetylcholine-induced contracture of the frog rectus. On the sciatic gastrocnemius preparation of the cat the compounds had a twofold action, consisting of an initial transient, and a delayed but more sustained, block of neuromuscular transmission. Bretylium and guanethidine had no immediate effect on the response of the muscle to direct stimulation, though a weak delayed depressant effect was observed. Intravenous injection of these compounds produced a flaccid paralysis in pigeons. Possible mechanisms of the neuromuscular blocking action of bretylium and guanethidine are discussed. Inhibition of acetylcholine release from motor nerve endings resulting from local anaesthesia by these compounds is suggested as a possible mechanism of their neuromuscular blocking action.

Two new antihypertensive drugs, bretylium and guanethidine, have recently been introduced in therapeutics. During the clinical use of these drugs, muscle weakness and fatigue have been reported as side-effects (Campbell & Montuschi, 1960; Evanson & Sears, 1960; Dollery, 1961; Page, 1961).

Boura & Green (1959) showed that bretylium blocks the contractile response of the rat phrenic nerve diaphragm preparation to indirect stimulation. They also reported respiratory paralysis and inhibition of the response of gastrocnemius muscle to indirect stimulation in cats anaesthetized with chloralose. Sensitivity to direct stimulation was found to be unaltered.

Vernikos-Danellis & Zaimis (1960), however, failed to observe any neuromuscular blocking action with either of the drugs in the tibialis anterior sciatic nerve preparation of cats, but demonstrated a very slow decrease in the maximal twitch tension of the muscle over a period of 2 to 3 hr. Electromyograms recorded from tibialis anterior muscles of these animals showed a myopathic pattern.

The present study was undertaken to investigate the neuromuscular blocking actions of bretylium and guanethidine.

METHODS

Rat phrenic nerve diaphragm preparation

The method was essentially similar to that described by Bülbring (1946). Rats weighing between 110 and 150 g were used. Unless otherwise specified all experiments were done at room temperature which ranged between 35 and 39° C. In all, 30 preparations were used.

For indirect stimulation the phrenic nerve was stimulated every 10 sec with single rectangular pulses of 4 V and 0.5 msec duration. For direct stimulation the tendinous portion of the muscle was connected to the writing lever by a thin copper wire which served as one electrode, the other electrode being fixed at the base of the fan-shaped diaphragm muscle. The muscle was stimulated every 10 sec with single rectangular pulses of 100 V and 5.0 msec duration.

In some experiments complete block to indirect stimulation was produced by tubocurarine (1×10^{-6}) before direct stimulation was begun.

In experiments designed to study the effect of lowering the temperature of the bath fluid on drug action, the temperature was maintained at the desired level ($\pm 1°$ C) and the muscle was allowed to acclimatize at each temperature for 20 min before the drugs were added.

Sciatic gastrocnemius preparation of cat

Eighteen cats weighing between 2 and 3 kg were anaesthetized with intravenous chloralose (90 mg/kg). Contractions of the gastrocnemius muscle were recorded isometrically as described by Burn, Finney & Goodwin (1952). For indirect stimulation the peripheral end of the cut sciatic nerve was stimulated every 10 sec with rectangular pulses of 5 V and 0.5 msec duration. For direct stimulation single rectangular pulses of 120 V and 5 msec duration were applied to the muscle every 10 sec by means of a bipolar electrode. Control responses to direct stimulation were taken after complete paralysis to indirect stimulation.

Retrograde injections were made into the cannulated contralateral common iliac artery. Heparin (10 mg/kg) was administered just after cannulation.

Frog rectus

Frogs were kept in a refrigerator at 10° C for about 24 hr, since muscles from such frogs gave more consistent responses to acetylcholine. The response of the muscle to acetylcholine chloride 0.6×10^{-6} to 1.2×10^{-5} was recorded for 90 sec every 10 min. When the response to a fixed dose of acetylcholine was reproducible the compounds under study were added to the bath for a specified period before the addition of acetylcholine and the change in the response to acetylcholine determined. Twelve preparations were used in all.

Pigeons

Drug solutions were injected intravenously into pigeons to study the paralytic effect on skeletal musculature. The criterion of paralysis was inability of the pigeon to stand on its legs. The slopes of the log dose-probit effect curves, the median paralytic dose (PD50), and the standard error of the PD50 were calculated as described by Burn *et al.* (1952), employing at least 15 pigeons for each determination.

Drugs

Bretylium tosylate, guanethidine sulphate, physostigmine salicylate, d-tubocurarine chloride and decamethonium iodide were used throughout these experiments. The doses or concentrations refer to the salts.

RESULTS

Phrenic nerve diaphragm preparation

Bretylium and guanethidine (1×10^{-4} to 3.3×10^{-4}) inhibited the contractile response of the diaphragm muscle to indirect stimulation ; the degree of block was proportional to the dose of the drugs. The blocking action was manifest immediately after the addition of the drugs ; complete recovery was obtained within 30 to 40 sec of the removal of the compounds from the bath. The compounds had no effect on the response of the muscle to direct stimulation, which was effective even after the compounds had produced complete block to indirect stimulation (Fig. 1). Likewise, in experiments where curarized preparations were used bretylium and guanethidine had no action on the response of the muscle to direct stimulation.

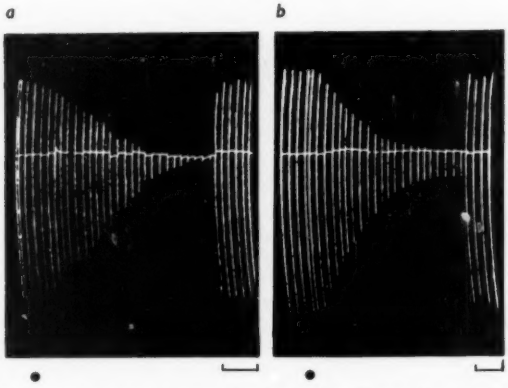

Fig. 1. Rat phrenic nerve diaphragm. Effect (at dots) of bretylium 3.3×10^{-4} (*a*) and guanethidine 3.3×10^{-4} (*b*) on the responses to indirect stimulation (4 V, 0.5 msec, 6/min). The period marked by bar indicates direct stimulation (100 V, 5 msec, 6/min) in presence of drugs.

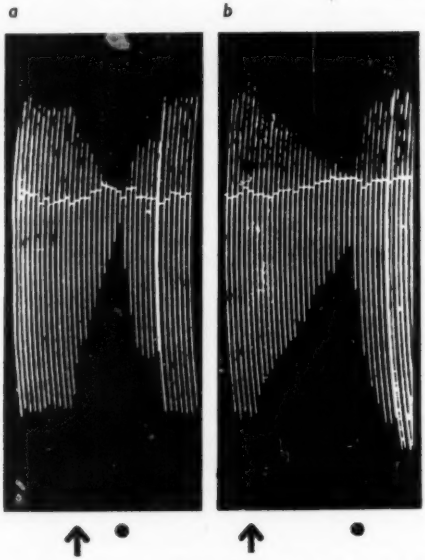

Fig. 2. Rat phrenic nerve diaphragm: indirect stimulation (4 V, 0.5 msec, 6/min). Effect (at dots) of potassium chloride (1.6 mg/ml.) on the blocking action of bretylium 3.3×10^{-4} (*a*) and guanethidine 3.3×10^{-4} (*b*) (at arrows).

Potassium chloride (1.6 mg/ml.) antagonized the blocking action of bretylium and guanethidine to indirect stimulation (Fig. 2). The block was not antagonized by physostigmine (3 μg/ml.).

Both tubocurarine and decamethoniun acted additively with bretylium or guanethidine (Fig. 3).

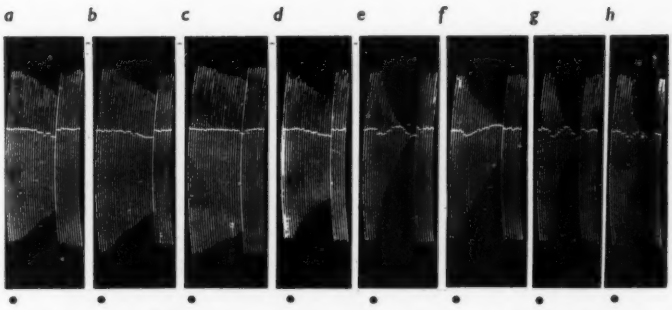

Fig. 3. Rat phrenic nerve diaphragm: indirect stimulation (4 V, 0.5 msec, 6/min). Effect of (at dots): (*a*) tubocurarine, 0.4×10^{-6}; (*b*) decamethonium, 3.3×10^{-5}; (*c*) bretylium, 2.3×10^{-4}; (*d*) guanethidine, 3.3×10^{-4}; (*e*) bretylium, 2.3×10^{-4}, plus tubocurarine, 0.4×10^{-6}; (*f*) bretylium, 2.3×10^{-4}, plus decamethonium 3.3×10^{-5}; (*g*) guanethidine, 3.3×10^{-4}, plus tubocurarine, 0.4×10^{-6}; (*h*) guanethidine, 3.3×10^{-4}, plus decamethonium, 3.3×10^{-5}.

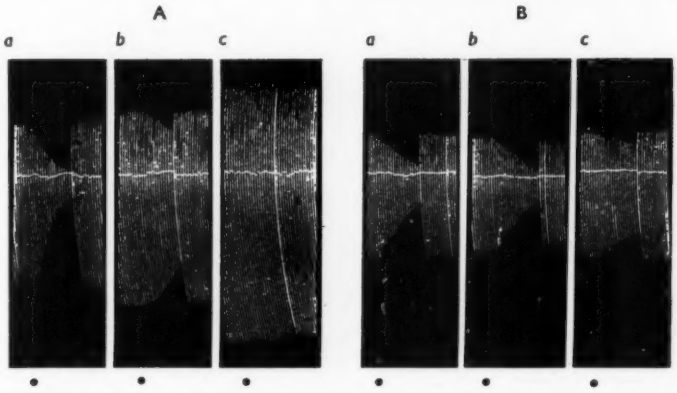

Fig. 4. Rat phrenic nerve diaphragm: indirect stimulation (4 V, 0.5 msec, 6/min). Effect (at dots) of bretylium 2.3×10^{-4} (A), and guanethidine 3.3×10^{-4} (B) at 36° C (*a*), 30° C (*b*), and 25° C (*c*).

The blocking action of bretylium and guanethidine was decreased when the temperature was lowered from 36° C to 25° C (Fig. 4).

Sciatic gastrocnemius preparation of cat

Bretylium and guanethidine (5 to 40 mg) injected intra-arterially produced a transient block, of varying degrees, of the response of the muscle to indirect stimulation; the drugs, however, had no immediate effect on the response of the muscle to direct stimulation (Fig. 5). In all but three experiments the initial transient block was followed after 15 to 60 min by a delayed block which progressed to completion within 30 to 290 min (Fig. 6). No recovery was seen even after 2 to 3 hr. After the response of the muscle to indirect stimulation was completely blocked, direct

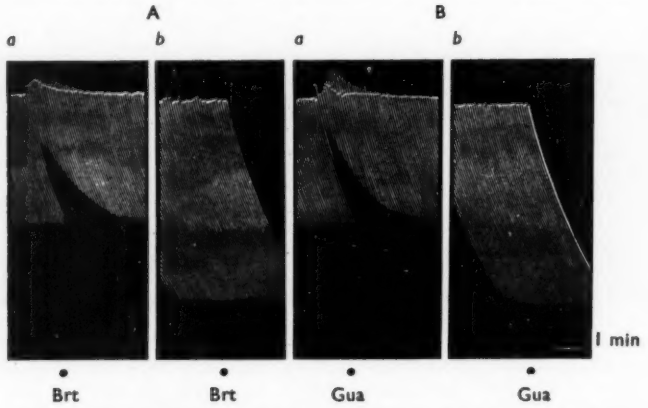

Fig. 5. Sciatic gastrocnemius preparation of cat. Chloralose anaesthesia. (*a*) Indirect stimulation (5 V, 0.5 msec, 6/min), and (*b*) direct stimulation (120 V, 5.0 msec, 6/min). Effect (at dots) of intra-arterial injections of bretylium 10 mg in A and guanethidine 10 mg in B.

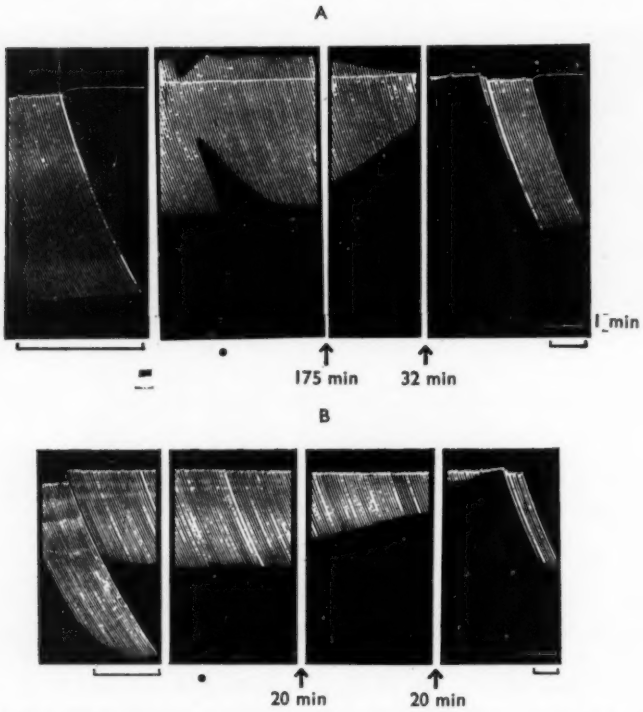

Fig. 6. Sciatic gastrocnemius preparation of cat. Indirect stimulation (5 V, 0.5 msec, 6/min). Effect of intra-arterial injections (at dots) of bretylium 40 mg (A) and guanethidine 20 mg (B). The period marked by bar indicates direct stimulation (120 V, 5 msec, 6/min).

stimulation was still effective, though less so than at the beginning of the experiment (Fig. 6). The delayed block was not antagonized by physostigmine (200 μg). Only in the initial stages could the block be temporarily reversed by potassium chloride (50 mg). Calcium chloride (50 mg) temporarily antagonized the delayed block produced by bretylium but not that elicited by guanethidine. Neuromuscular blocking action of tubocurarine (200 μg) was enhanced both in magnitude and in duration when tubocurarine was preceded by a small amount of bretylium (5 mg) while guanethidine (5 mg) increased only the duration of the block. The neuromuscular blocking action of decamethonium (30 μg) was not modified by either of the drugs.

Frog rectus

Bretylium and guanethidine (0.6×10^{-6} to 3×10^{-4}) had no inhibitory action on acetylcholine-induced contracture of the frog rectus. On the other hand, the compounds potentiated the stimulant action of acetylcholine (Fig. 7).

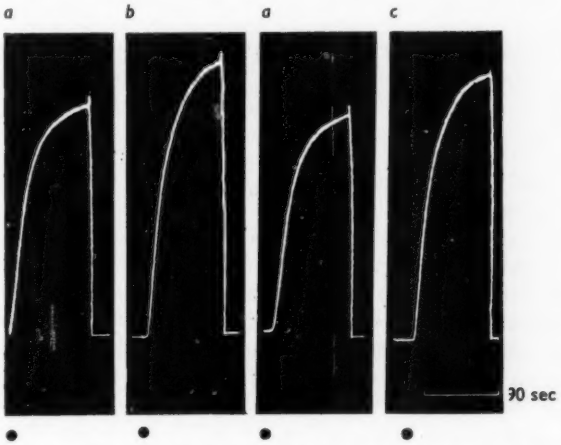

Fig. 7. Frog rectus. Effect of acetylcholine 0.6×10^{-6} (at dots). (*a*) Control responses; (*b*) and (*c*) 1 min after bretylium 0.6×10^{-6} and guanethidine 5×10^{-6} respectively.

Pigeons

Transient flaccid paralysis was observed in pigeons after the intravenous administration of bretylium and guanethidine. The PD50 values for bretylium and guanethidine were 23.1 ± 1.06 mg/kg and 10.86 ± 1.04 mg/kg respectively.

DISCUSSION

The results of our experiments show that both bretylium and guanethidine block transmission across the neuromuscular junction. On the sciatic gastrocnemius preparation of the cat the compounds had a twofold action, consisting of an initial transient, and a delayed but more sustained, block of neuromuscular transmission. The initial blocking action was short-lived and was not studied in detail. The

delayed blocking action and the blocking action on the rat phrenic nerve diaphragm preparation were studied in detail and are discussed.

Neuromuscular blocking agents in common use can be divided into two groups on the basis of the mechanism by which they act ; those which block by competition and those which block by depolarization. Each of these two types of neuromuscular block are pharmacologically well characterized (Paton & Zaimis, 1952 ; Brücke, 1956). An analysis of the neuromuscular blocking action of bretylium and guanethidine on the two nerve-muscle preparations used indicates that the block produced by these compounds belongs to neither of these two types. Antagonism of the blocking action of these compounds by potassium in rat phrenic nerve diaphragm and in the initial stages in sciatic gastrocnemius preparation indicates a possible similarity with the non-depolarizing type of block. However, the blocking action of these compounds is not antagonized by doses of physostigmine which completely antagonize tubocurarine block. Holmes, Jeden & Taylor (1951) and Bigland, Goetzee, Maclagan & Zaimis (1958) have shown that the neuromuscular blocking action of tubocurarine decreases and that of the decamethonium type of drugs increases with lowering of temperature. If this difference is taken as a criterion of the two types of neuromuscular block, the action of bretylium and guanethidine on the phrenic nerve diaphragm preparation seems to be of a non-depolarizing type.

Straughan (1961) has shown that procaine produces neuromuscular block by inhibiting the release of acetylcholine from motor nerve endings. In view of the reported local anaesthetic activity of bretylium and guanethidine (Boura & Green, 1959 ; Green, 1960), a similar explanation could be suggested for the blocking action of these compounds. This view is supported by the fact that, like procaine (Dixit, Gulati & Gokhale, unpublished observations), bretylium and guanethidine act additively with both tubocurarine and decamethonium and that their blocking action is not antagonized by physostigmine. Small amounts of bretylium or guanethidine potentiated the neuromuscular block produced by tubocurarine in the sciatic gastrocnemius preparation of cat, but did not modify the action of decamethonium, an observation consistent with an inhibition of release of transmitter from motor nerve endings. Moreover, absence of an inhibitory effect on acetylcholine-induced contracture of the frog rectus indicates a presynaptic site of blocking action of these drugs. With bretylium the local anaesthetic action is of considerable duration (Boura & Green, 1959), and this might explain the long duration of the delayed block seen in the sciatic gastrocnemius preparation of the cat.

Since the response of the gastrocnemius muscle to direct stimulation was not as fully effective as at the beginning of the experiment, after complete block to indirect stimulation was produced by bretylium or guanethidine, it is possible that these compounds also produce a delayed direct depression of muscle contractility.

The authors gratefully acknowledge the generous gifts of bretylium tosylate and decamethonium iodide by Burroughs Wellcome & Co., and guanethidine sulphate by Ciba (Basle). Thanks are also due to Dr J. D. Pathak, Dean, Medical College, Baroda, for providing facilities to carry out the work.

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THE RESERPINE-TREATED CAT

BY

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In cats, 24 hr after the administration of 1 mg/kg of reserpine, it was found that (*a*) the heart is in failure; (*b*) the sensitivity of the peripheral vessels to adrenaline, noradrenaline and isoprenaline, administered intravenously or close-arterially, is decreased; (*c*) any blood pressure changes are, as a rule, secondary to changes in heart contraction; and (*d*) the peripheral blood flow passively follows the blood pressure changes. Furthermore, any improvement of the circulation at this stage was found to be almost exclusively the result of an amelioration in the force of cardiac contraction.

Reserpine is known to reduce or deplete the various tissues of their catecholamine content, and its pharmacological actions on circulation have been, as a rule, attributed to this depletion (Burn & Rand, 1958; Axelrod & Tomchick, 1960). Because of this, during the last few years the cardiovascular system of animals pretreated with reserpine has been extensively used for the pharmacological analysis of the action of a multitude of drugs. In most studies the cat has been a favourite experimental animal, and, in order to "reserpinize" it, doses of at least 1 mg/kg of the drug have usually been used. The severe clinical condition, however, in which the cats are found 24 hr after the administration of such a dose of reserpine and the marked degenerative changes of the heart muscle revealed by histological examination (Zaimis, 1961) were considered unlikely to be the result only of a depletion, however complete, of the animal's catecholamines. Therefore, experiments were undertaken in which the various components of the cardiovascular system could be studied simultaneously *in vivo*.

METHODS

Both the control and the reserpine-treated cats were anaesthetized with a mixture of chloralose (80 mg/kg) and pentobarbitone (6 mg/kg) injected into the cephalic vein of the fore limb or the saphenous vein of the hind limb. The addition of pentobarbitone prevented the initial stage of excitement which normally follows the intravenous injection of chloralose alone. Because of their poor condition, the reserpine-treated animals had to be anaesthetized very slowly.

Recording of the venous outflow from the hind limb. An incision was made in the skin of the medial surface of the thigh, and all branches of both femoral artery and vein down to the popliteal space, including the saphenous vein, were ligated and cut, with the exception of the artery chosen for the intra-arterial administration of the drugs. In order to maintain a regular blood-flow through the limb, the muscles were stimulated indirectly by the application of electrical shocks to the sciatic nerve at a frequency of 6 stimuli/min. Heparin

(1,000 units/kg) was administered intravenously 10 min before any vessels were opened. The method described by Hilton (1952, 1953) was used for recording the venous outflow. According to this method the femoral vein is cannulated, and the blood is allowed to pass through a drop chamber and then straight back into the animal. The drugs were injected *close-arterially* by means of a cannula made from a no. 18 hypodermic needle tied into the cut end of a branch of the femoral artery, usually the small artery supplying the gracilis muscle.

Direct measurement of changes in cardiac contractile force. The chest was opened and the pericardium incised and secured to the chest wall. The lungs were ventilated throughout by means of a Starling pump. The force of ventricular contraction was measured with a strain gauge arch and the signals from the arch were fed into an ultra-violet polygraph (New Electronics Products), and recorded on moving paper. The strain gauge arch was attached directly to the muscle of either the right or the left ventricle by cotton sutures so placed as to avoid occlusion of any major branch of a coronary vessel. The strain gauge technique is a safe and practical method for assessing the inotropic effects of various drugs (Cotten & Bay, 1956). As a rule, no blood pressure irregularities appeared after the arch had been attached to the heart, and, furthermore, in control experiments it was found that both force of heart contraction and blood pressure remained steady for periods as long as 24 hr.

Drugs. Adrenaline acid tartrate, noradrenaline acid tartrate, isoprenaline hydrochloride and tyramine hydrochloride were used throughout these experiments, and are referred to as adrenaline, noradrenaline, isoprenaline and tyramine, respectively. Phenoxybenzamine (dibenyline hydrochloride) was dissolved in propylene glycol (1%), and immediately before administration a 1:10 dilution in physiological saline was made. A 2.5 mg/ml. solution of reserpine (Serpasil; Ciba) was used as such or further diluted with physiological saline. The drug was administered either subcutaneously or intraperitoneally. Most animals were given a single dose of 1 mg/kg on the day before the experiment. A few received the same amount of the drug spread over two or three days.

RESULTS

Clinical picture

Twenty-four hours after the administration of 1 mg/kg of reserpine the cats, although not paralysed, lay immobile, unresponsive to sound, and appeared almost lifeless. When handled and gently caressed, however, they opened their eyes and with great difficulty succeeded in making some movements, while purring and yet crying simultaneously. The temperature of the room in which the cats were kept was about 20° C, and physiological saline and 5% dextrose were administered orally and intravenously twice daily. In these animals, despite all precautions, body temperature tended to fall, respiration was depressed, blood pressure low and the heart rate markedly reduced. There was a loss of body weight, possibly because the animals neither ate nor drank and, in addition, suffered from marked diarrhoea. In other words, a cat treated in this way is a very sick animal and presents a picture of great misery very similar in many ways to that of a patient in advanced shock (for example, late stages of acute circulatory failure) and definitely very much unlike the picture of an animal under the influence of an ordinary central depressant drug.

Cardiovascular studies

Peripheral vessels. In the first group of experiments the simultaneous recording of blood pressure and peripheral blood flow revealed that in the animals treated with reserpine the sensitivity of the peripheral vessels to adrenaline, noradrenaline and isoprenaline, administered either intravenously or close-arterially, was decreased.

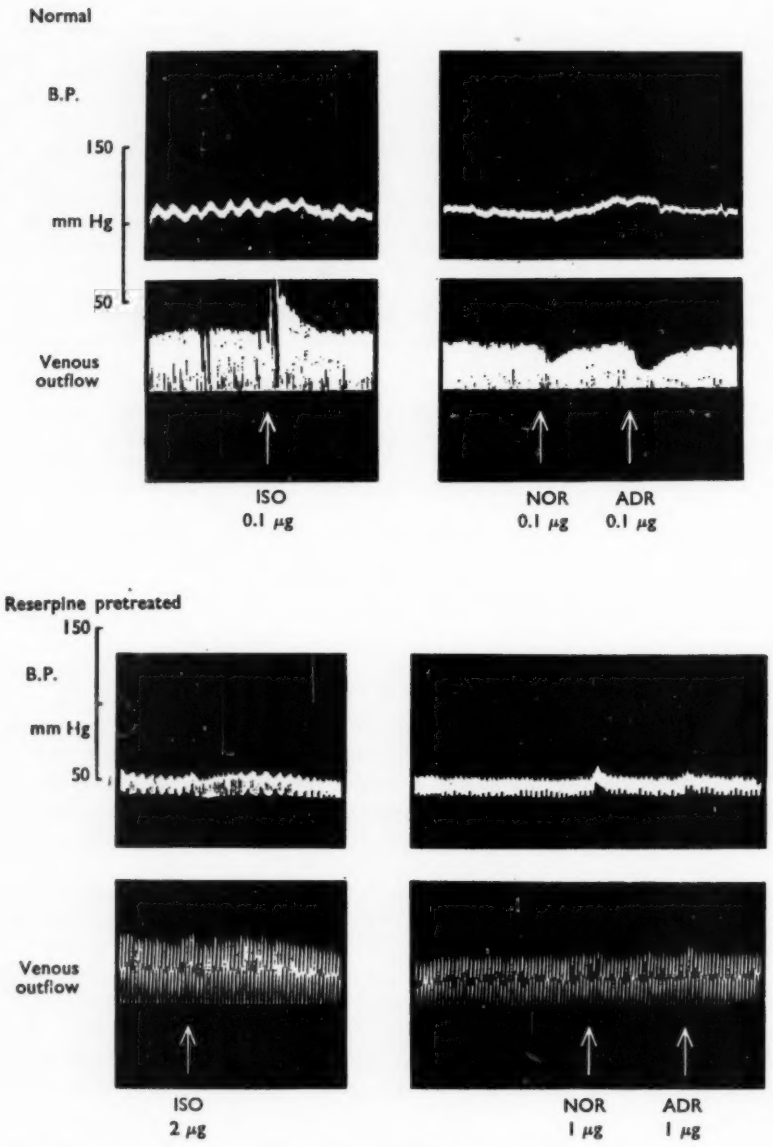

Fig. 1. Blood pressure and venous outflow from a hind limb recorded simultaneously. Isoprenaline (ISO), noradrenaline (NOR) and adrenaline (ADR) were administered by close-arterial injection. Upper tracing: control cat. Lower tracing: reserpine-treated, 1 mg/kg, on the day before the experiment.

As a rule in the normal cat after a close-arterial injection of 0.1 μg of isoprenaline, noradrenaline or adrenaline there is no change in general blood pressure. However, as Fig. 1 shows, this dose of isoprenaline produced a local vasodilatation accompanied by an increase in blood flow, while after noradrenaline or adrenaline, because of the local vasoconstriction, the blood flow was decreased. In contrast, in animals pretreated with I mg/kg of reserpine similar or even larger doses of the catecholamines appeared ineffective or gave rise to very reduced responses (Fig. 1). This decreased sensitivity of the peripheral vessels was found also in experiments in which larger doses of the same drugs were administered intravenously. Fig. 2 illustrates typical differences between a normal and a reserpine-treated animal. In the normal animal, an intravenous dose of 10 μg of noradrenaline was followed by a large increase in blood pressure and a simultaneous decrease in the peripheral blood flow. A similar effect was produced by tyramine. In the same preparation, 10 μg of isoprenaline produced the usual fall in blood pressure and a simultaneous increase in the peripheral blood flow. The picture in the reserpine-treated animal was quite different: the peripheral blood flow appeared to follow passively the blood pressure changes, as if the vessels had lost their ability to participate actively in the control of the circulation. There was no sign of either vasoconstriction following the administration of noradrenaline, adrenaline or tyramine, or of vasodilatation following that of isoprenaline. These results suggested that, in the animals treated with reserpine, vasoconstriction could play only a very small rôle, if any, in the rise of blood pressure elicited by noradrenaline or adrenaline.

Heart. In order to elucidate the mechanism of the pressor response to catecholamines in the reserpine-treated cats, experiments were performed in which the force of the cardiac contraction and changes in blood pressure and blood flow could be recorded simultaneously. When the chest was opened, the heart was found to be enlarged, flabby and soft, and the superior and inferior venae cavae and their tributaries engorged. At this stage the right side of the heart was, as a rule, more affected than the left. Moreover, in several experiments in which absolute calibration of the force of cardiac contraction (in grams) had been done, it was found that in the reserpine-treated animals the force of contraction was about one-half or one-third of that measured in the normal cat. Thus the weak contraction of the myocardium together with the macroscopic picture suggested strongly that these hearts were in failure.

The results obtained from the simultaneous recording of blood pressure, force of cardiac contraction, and peripheral blood flow showed that in the reserpine-treated animal any blood pressure changes were, as a rule, secondary to changes in cardiac contraction. Fig. 3 illustrates an experiment in which noradrenaline was injected before and after the administration of 4 mg/kg of dibenyline—a dose large enough to abolish in the normal animal the vasoconstrictor action of noradrenaline. In cats treated with reserpine, 2 hr following the administration of dibenyline, noradrenaline produced the same changes in both the force of cardiac contraction and in blood pressure. Obviously in this animal the rise in blood pressure elicited by noradrenaline was secondary to the former effect. In other experiments, after dibenyline the rise in blood pressure elicited by noradrenaline appeared to be

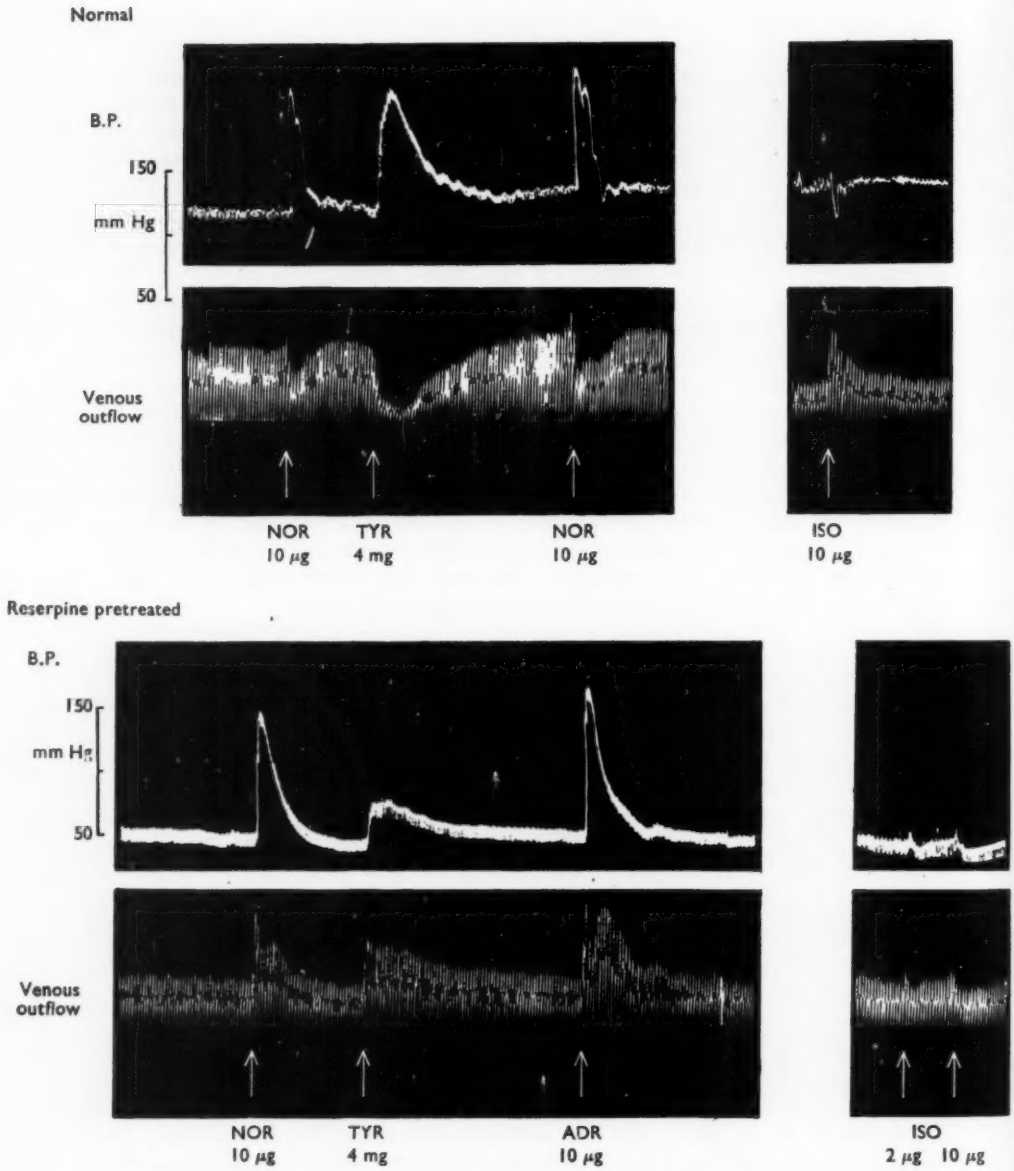

Fig. 2. Blood pressure and venous outflow from a hind limb recorded simultaneously. Noradrenaline (NOR), tyramine (TYR), isoprenaline (ISO) and adrenaline (ADR) were administered intravenously. Upper tracing: control cat. Lower tracing: reserpine-treated, 1 mg/kg, on the day before the experiment.

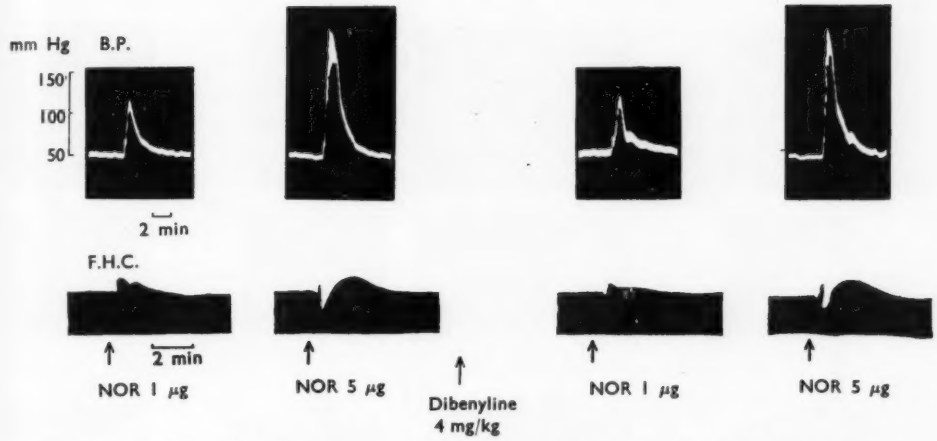

Fig. 3. Cat, 2.9 kg. Reserpine-treated, 0.5 mg/kg, for 2 days before the experiment. Upper tracing: blood pressure. Lower tracing: precise reproduction of actual recordings of ventricular contractile force. Force of heart contraction (F.H.C.). The last 2 doses of noradrenaline (NOR) were injected 2 hr after the administration of dibenyline.

reduced. However, the blood pressure reduction was always accompanied by a reduced effect of noradrenaline on the force of cardiac contraction. Fig. 4 illustrates such an experiment. After dibenyline, noradrenaline produced a smaller positive inotropic effect and at the same time a smaller rise in blood pressure. Once more the peripheral blood flow passively followed the blood pressure changes.

Further proof that the hearts of animals treated with reserpine were in failure was obtained from experiments in which the effect of ouabain was tested. In control cats a dose of 50 to 100 μg of ouabain produced very little or no change at all in either the force of cardiac contraction or in the blood pressure. In contrast, in the reserpine-treated animals after ouabain a gradual increase in the amplitude of cardiac contraction occurred, accompanied by a simultaneous rise in blood pressure. Furthermore, in the presence of ouabain the positive inotropic effect of various drugs, which in the animals treated with reserpine became less effective or depressant to the heart, was partially restored. For example, in the experiment just described and illustrated in Fig. 4, after 50 μg of ouabain the positive inotropic effect of noradrenaline increased, and as a result a greater rise in blood pressure was produced. This beneficial effect of ouabain was seen also in experiments in which the effect of tyramine was studied. In control animals, the administration of tyramine was accompanied by a large rise in blood pressure and a marked increase in the force of cardiac contraction. In the cats treated with reserpine, tyramine depressed cardiac contraction and the rise in blood pressure was markedly reduced. After ouabain, however, the depression of cardiac contraction produced by tyramine was prevented and the rise in blood pressure increased. Fig. 5 illustrates these effects.

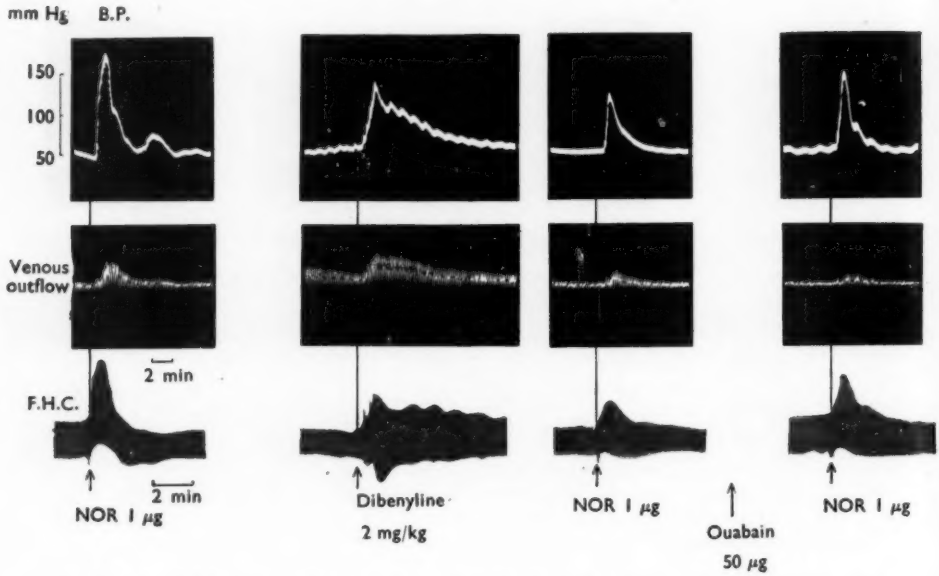

Fig. 4. Cat, 1.8 kg. Reserpine-treated, 1 mg/kg, the day before the experiment. Upper tracing: blood pressure. Middle tracing: venous outflow from a hind limb. Lower tracing: precise reproduction of actual recordings of ventricular contractile force. The second dose of noradrenaline (NOR) was injected 1 hr after the administration of dibenyline. The third dose of noradrenaline was injected 20 min after the administration of ouabain.

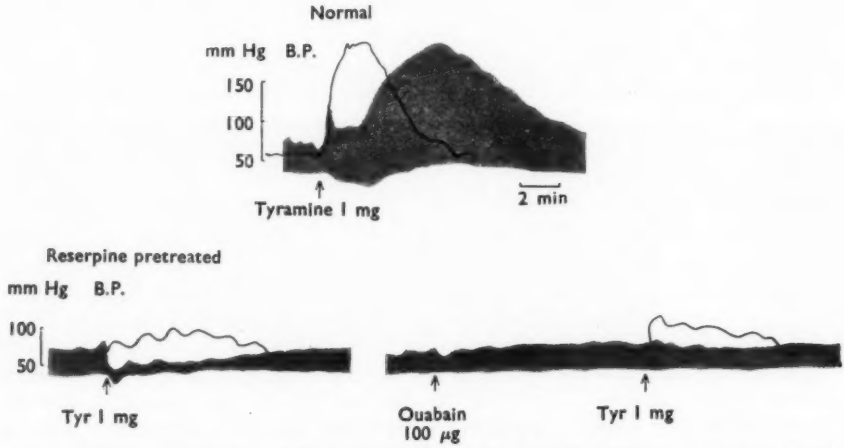

Fig. 5. Upper tracing: cat, 1.6 kg.; control. Lower tracing: cat, 2.7 kg.; reserpine-treated, 1 mg/kg. the day before the experiment. Precise reproductions of blood pressure changes and of actual recordings of ventricular contractile force.

It is well known that in the normal cat adrenaline, noradrenaline and isoprenaline increase the force of cardiac contraction, isoprenaline being the most powerful of the three (Fig. 6). In the reserpine-treated animal, however, the picture was completely different. Noradrenaline then produced, as a rule, the greatest inotropic effect, adrenaline was second, and isoprenaline, according to the condition of the heart, produced an increase, a depression or no effect. Fig. 6 illustrates an experiment in which 1 μg of isoprenaline, injected intravenously into an animal which had received the day before the experiment 1 mg/kg of reserpine, produced a marked depression of cardiac contraction.

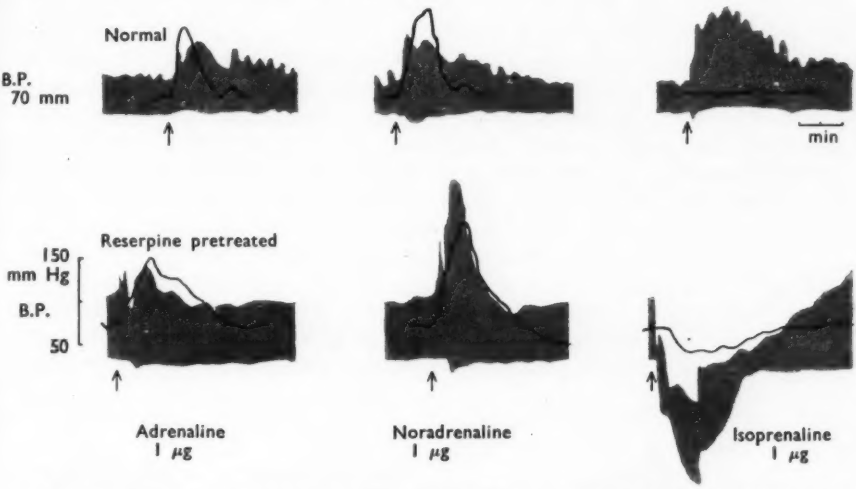

Fig. 6. Upper tracing: cat, 2.1 kg.; control. Lower tracing: cat, 2.7 kg.; reserpine-treated, 1 mg/kg, the day before the experiment. Precise reproductions of blood pressure changes and of actual recordings of ventricular contractile force.

Ouabain once more improved the response of the heart to isoprenaline. Fig. 7 illustrates an experiment in which isoprenaline produced very little change in the force of the heart contraction. Following 100 μg of ouabain, however, its positive inotropic effect improved, and there was a simultaneous rise in blood pressure.

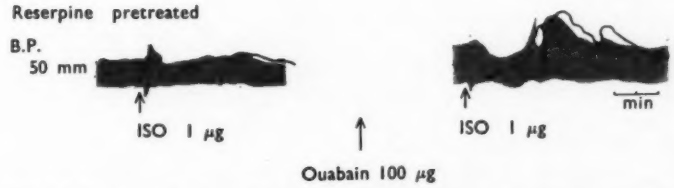

Fig. 7. Cat, 1.8 kg. Reserpine-treated, 1 mg/kg, the day before the experiment. Precise reproductions of blood pressure changes and of actual recordings of ventricular contractile force. The second dose of isoprenaline (ISO) was administered 30 min after the injection of ouabain.

Such a rise in blood pressure always occurred when isoprenaline increased the force of cardiac contraction in a reserpine-treated animal. Again, this is an abnormal response, since the powerful inotropic effect which isoprenaline elicits in the normal cat is not usually accompanied by a rise in blood pressure. In contrast, in the animal treated with reserpine the blood pressure changes faithfully followed the effects of isoprenaline on the force of cardiac contraction, probably because in these animals the direct effect of the drug on the peripheral blood vessels had been almost abolished. Thus isoprenaline, when positively inotropic, produced a clear rise in blood pressure, lasting as long as its effect on the heart persisted. If there was no positive inotropic effect or if the drug depressed the heart muscle, the blood pressure was unaffected or actually lowered.

The fact that the heart is possibly one of the first targets in the activity of reserpine is clearly illustrated by an experiment performed on a cat which received for a period of two months a daily dose of 10 μg/kg of reserpine. At the end of the two months the general condition of this animal, its body temperature and its blood pressure appeared to be fairly normal. The cat was then anaesthetized and prepared for the simultaneous recording of blood pressure, blood flow and force of cardiac contraction. The results showed that, although the force of cardiac contraction appeared fairly normal, the sensitivity of the myocardium to both catecholamines and ouabain was very high. For example, all three catecholamines, particularly isoprenaline, produced good positive inotropic effects in a total dose of only 0.1 μg. Moreover, as Fig. 8 shows, the positive inotropic effect of isoprenaline was accompanied by a diphasic response of the blood pressure, a small fall followed by a

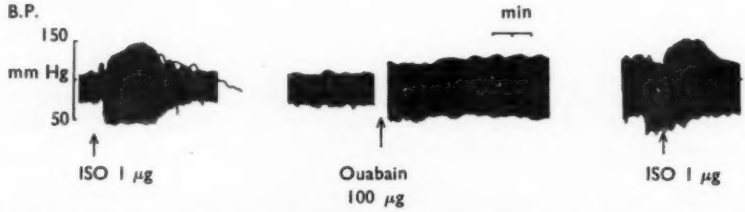

Fig. 8. Cat, 2.4 kg. Reserpine-treated, 10 μg/kg, for two months. Precise reproductions of blood pressure changes and of actual recordings of ventricular contractile force. The third section of the record was taken 20 min after the administration of ouabain and the fourth 15 min later.

rather pronounced and prolonged rise. Following the administration of 100 μg of ouabain, the force of cardiac contraction nearly doubled and there was a simultaneous substantial rise in blood pressure. The ouabain effect in this animal was far larger than that recorded previously in cats which had been treated with a dose of 1 mg/kg of reserpine and which at the time of the experiment were in obvious heart failure. Furthermore, following the administration of ouabain the features of cardiac insufficiency disappeared, the isoprenaline effect became normal, and its inotropic effect was accompanied once more by a fall and not a rise in blood pressure. Both these points are illustrated by Fig. 8. Thus, the results obtained

from this experiment, when put together, indicate (*a*) that the heart can be affected by even very small doses of reserpine administered over long periods of time; (*b*) that insufficiency of the myocardium may be present at a stage when obvious signs of heart failure are absent; and, finally, (*c*) that at this stage both catecholamines and ouabain exhibit powerful positive inotropic effects.

Heart rate. The reason for the rather puzzling behaviour of the catecholamines in animals treated with reserpine became apparent when heart rate changes were recorded at the same time as the other parameters. It was found that there was a close relationship between the inotropic effect of the drug and the ability of the failing heart to sustain an increased activity. In the treated as in the control animals, isoprenaline produced the most marked increase in heart rate; adrenaline came second, producing a smaller but still sustained tachycardia, while noradrenaline was the least effective in both magnitude and duration of the effect. The failing heart of the animal treated with reserpine, however, in contrast to the control one, proved unable to sustain an increased activity. Therefore the response to isoprenaline varied according to the condition of the heart at the moment of the drug administration. If very poor, the heart faced the abrupt increase in heart rate with a clear-cut depression in the force of its contraction. On the other hand, when failure was not very advanced, the cardiac contraction could still improve, but even then the response was always much smaller than that usually seen in the control heart. A similar variation, although less pronounced, was found with adrenaline. In the majority of experiments adrenaline did produce a positive inotropic effect. Occasionally, however, when heart failure was very advanced, the effect of adrenaline on the heart was depressant. Only the administration of noradrenaline was always followed by a positive inotropic effect.

DISCUSSION

The results just described show that, in cats, 24 hr after the administration of reserpine in a dose of about 1 mg/kg, the heart is in failure and the sensitivity of the peripheral vessels to adrenaline, noradrenaline and isoprenaline, administered intravenously or close-arterially, decreased. Furthermore, any blood pressure changes are, as a rule, secondary to changes in cardiac contraction and the peripheral blood flow passively follows the blood pressure changes. Moreover, any improvement of the circulation at this stage was found to be almost exclusively the result of an amelioration in the force of cardiac contraction. For example, ouabain increased the amplitude of the cardiac contraction and partially restored the positive inotropic effect of various drugs which in the animals treated with reserpine became ineffective or depressant to the heart. This was the case with both tyramine and isoprenaline. In other words, in a very short period of time, the cat treated with reserpine exhibited a most spectacular circulatory failure.

In the present experiments the doses of reserpine have been much larger than those used in therapeutics. However, there are several clinical reports of oedema and congestive failure related to the administration of relatively small doses of reserpine. Perera (1955) reported 5 cases which within a week after the daily administration of 0.4 mg of reserpine or 200 mg of raudixin developed oedema,

especially of the ankles, occasionally accompanied by moderate exertional dyspnoea. Within a week after the drug had been discontinued all manifestations of abnormal fluid retention disappeared, and during a period of observation of from 1 to 3 months after treatment no patient developed any recurrent oedema or cardiac symptoms. Later on reserpine was re-administered to one patient for one week and the same symptoms appeared again. Marley & Pare (1956) presented two cases of cardiac failure which occurred following the administration of reserpine, and very recently the *British Medical Journal* (1961, **i**, 1022) through an editorial article urged the clinicians to consider patients on reserpine as potentally "poor risks" when their cardiovascular system had to cope "with an increased load of work—for example, during labour, electro-shock therapy or anaesthesia and surgery."

Histological examination of the heart and other organs removed from animals pretreated for one or several days with a total dose of reserpine of 1 to 1.25 mg/kg revealed pronounced changes (Zaimis, 1961). The heart muscle showed extremely severe degenerative changes, the adrenal cortex a picture characteristic of severe stress, and the liver centrilobular (fatty) changes indicative of heart failure and increased venous pressure.

All these results put together indicate the remarkable toxicity of reserpine on the myocardium. With large doses, of the order of 1 mg/kg, the heart goes, in a few hours, into a most spectacular failure. Even with very small doses administered over a long period of time the myocardium is affected, but it appears that the changes become obvious only when a special demand is put upon the heart.

There is a striking similarity between the behaviour of a heart poisoned with a large dose of reserpine (1 mg/kg) and that developing in conditions in which the oxygen supply or the oxygen utilization of the myocardium is substantially decreased. In both instances the cardiac capacity for work declines rapidly and the heart slows down in order to do a given amount of work per unit of time more economically. However, as the heart tires and the functional capacity decreases its mechanical efficiency is diminished. To do the same amount of work the heart dilates continuously and finally failure of function sets in.

The mechanism by which reserpine gives rise to these toxic changes remains to be elucidated; probably the drug produces either a defect in the myocardial metabolism of various substrates or an abnormality of the contractile proteins. Meantime, one definite conclusion can be drawn: cats, pretreated with 1 mg/kg of reserpine, because of the acute circulatory failure, must be considered as most unreliable experimental tools.

Our thanks are due to the Medical Research Council (E.Z.) for a grant for technical assistance, and to the National Institutes of Health, U.S. Public Health Service [Grant B-2474 (CI)], for partially financing this research.

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IMMEDIATE EFFECTS OF CATECHOLAMINES ON PLASMA FIBRINOGEN IN CONSCIOUS AND ANAESTHETIZED DOGS

BY

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Adrenaline, noradrenaline, and isoprenaline were injected into dogs and the levels of plasma fibrinogen were estimated for up to 90 min. No changes in the concentrations of circulating plasma fibrinogen were found following the administration of any of the agents. These results do not support the view that hyperfibrinogenaemia occurs immediately following injection of adrenaline or that changes in fibrinogen concentration are related to alterations in blood pressure.

It has been reported that adrenaline produces a prompt and marked increase in the concentration of plasma fibrinogen in the dog (Rieker & Winters, 1931 ; Goreczky & Kováts, 1942). This hyperfibrinogenaemia occurred within 15 min following intravenous or subcutaneous injection of the drug. These results indicated the existence of a fibrinogen pool which was available for rapid mobilization by the autonomic nervous system. An inquiry into the mechanism of fibrinogen release from storage sites and the action of the common catecholamines seemed important in the light of other experiments which indicate that such compounds are not immediately effective in the rat (Henriques, Henriques & Levy, 1956). Thus, we have studied the response of dogs to the pure L-isomers of adrenaline and noradrenaline and to isoprenaline.

METHODS

Thirteen healthy mongrel dogs of both sexes, obtained from the pound, were used directly or kept for 27 to 55 days in individual cages in veterinary quarters. These latter animals were fed once daily a diet consisting of a commercial dog food mixed with cooked lean beef, and water was available at all times. The dogs were allowed to exercise daily.

The drugs employed in this study were (−)-adrenaline bitartrate (100 μg/kg in 1 to 2.5 ml. of solution), obtained from the Aldrich Chemical Co., Milwaukee 10, Wisconsin (m.p. 144° C), or the Winthrop Laboratories, New York, 18, New York (m.p. 149–150° C), and (−)-noradrenaline bitartrate (Levophed) (8 μg/kg in 0.08% solution) and isoprenaline hydrochloride (Isuprel) (5 μg/kg in a 0.05% solution), obtained from the Winthrop Laboratories, New York 18, New York. The diluent used was 0.85% sodium chloride solution. The drugs were tested in dogs which had received no anaesthetic and in others which were anaesthetized with pentobarbitone sodium or with a combination of thiopentone sodium and chloralose. In the latter procedure, thiopentone sodium (15 mg/ml.) was injected by vein until the animal became quiet enough to be given 100 mg/kg of chloralose by vein, which was dissolved in a minimum volume (usually 150 ml.) of warmed 0.85% sodium chloride solution. Each anaesthetized animal was connected through a tracheal cannula to a positive pressure respiration pump (Harvard Apparatus Company) and pulmonary ventilation was adjusted while monitoring arterial blood *p*H. Blood pressure was recorded from the left common carotid

artery by means of a Sanborn pressure transducer coupled to a Sanborn photographic oscillograph.

The right femoral triangle was dissected and the femoral artery was exposed distally for about 15 cm. Blood samples were withdrawn from this artery through 20-gauge needles by taking the first sample from the most distal segment and subsequent samples from more proximal segments. The drugs were injected into the right femoral vein. Blood pressure and heart rate responses indicated that the doses used were effective. In one animal, L-adrenaline bitartrate (97 μg/kg in 9.7 ml. solution) was infused over a 5 min period. Blood samples were withdrawn from unanaesthetized dogs via the left and right antecubital veins, and drugs were injected by the same route as well as by the subcutaneous route. All samples were immediately decalcified by mixing 9 parts of blood with one part of cold 4.0% trisodium citrate solution. This mixture was kept for 90 min or less in an ice-water bath and then centrifuged. Fibrinogen was estimated according to a semi-micro technique. Plasma (0.2 ml.) was clotted with thrombin (10 u.) in the presence of ice-cold phosphate buffer, *p*H 6.4. The resulting fibrin was wound out after 30 min incubation in an ice-water bath and dissolved in 0.1 N sodium hydroxide. Colour was developed with Folin-Ciocalteu reagent using L-tyrosine as a standard. An appropriate tyrosine-fibrinogen conversion factor (Johnston & Gibson, 1938) enabled a comparison to be made of results obtained by the Folin-Ciocalteu method with those obtained by a Kjeldahl technique. Triplicate analyses were performed on each sample.

RESULTS

Upon injection of 100 μg/kg of adrenaline, blood pressure in 5 animals changed from a mean of 145 mm mercury to a mean of 276 mm mercury and returned to the control value within 10 min. Average heart rate changed from 162 beats/min to 255 beats/min and returned to control values within 30 min. The effect of the injection of 8 μg/kg of noradrenaline was established in experiment 1 ; the mean blood pressure changed from 123 mm mercury to 178 mm mercury and returned to normal within a 30 min period. There was no change in heart rate. The effect of the injection of 5 μg/kg of isoprenaline was established in experiment 3 ; the mean blood pressure fell from 107 mm mercury to 48 mm mercury and returned to normal within a 10 min period. Heart rate increased from 156 to 312 beats/min and returned to near control value within 20 min.

Data for fibrinogen levels for each experiment are presented to emphasize the constancy of the results obtained. (Experiments are not numbered consecutively.) Fig. 1 presents the fibrinogen values obtained from animals which were injected with L-adrenaline. In experiments 4 and 5 the anaesthetic used was pentobarbitone

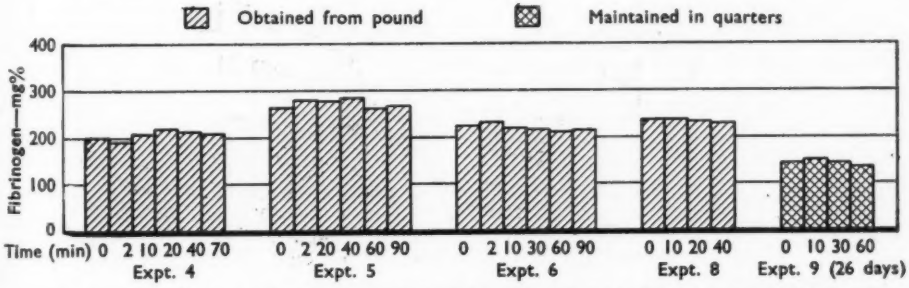

Fig. 1. Plasma fibrinogen levels in anaesthetized dogs after administration of adrenaline bitartrate, 100 μg/kg, in 1 to 2.5 ml. of solution.

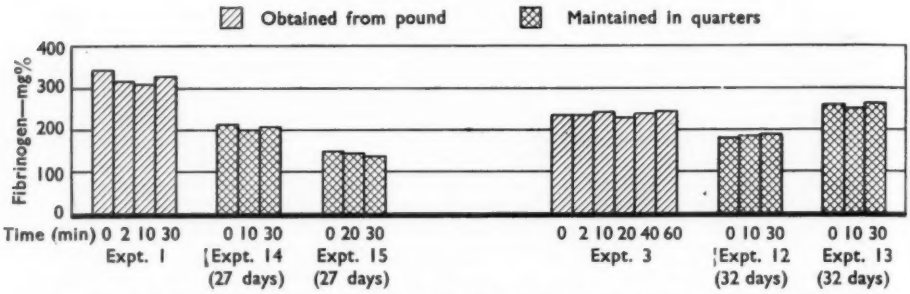

Fig. 2. Plasma fibrinogen levels in anaesthetized and conscious dogs after administration of noradrenaline, 8 μg/kg (expts. 1, 14 and 15), and isoprenaline, 5 μg/kg (expts. 3, 12 and 13).

sodium; in experiments 6, 8 and 9 the anaesthetic agents were thiopentone sodium and chloralose. Fig. 2 shows the results obtained when noradrenaline and isoprenaline were injected. The anaesthetic used in experiments 1 and 3 was pentobarbitone sodium; no anaesthetic was used in experiments 12, 13, 14 and 15. Inspection of the control values revealed that with one exception fibrinogen was lower in dogs that had been housed in veterinary quarters (mean is 185 mg/100 ml.) than in those obtained directly from the pound (mean is 249 mg/100 ml.).

In two unanaesthetized dogs, a mixture of adrenaline and noradrenaline was injected subcutaneously (Fig. 3). Dosage given was 80 μg/kg of adrenaline and 20 μg/kg of noradrenaline.

Thus it was found that injections of adrenaline, noradrenaline, isoprenaline or a combination of adrenaline-noradrenaline produced no immediate effects on plasma fibrinogen, although the doses employed produced profound circulatory changes.

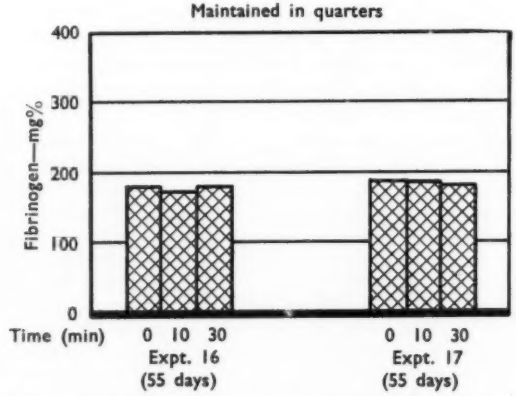

Fig. 3. Plasma fibrinogen levels in conscious dogs, maintained in quarters, after administration of a mixture of adrenaline, 80 μg/kg, and noradrenaline, 20 μg/kg.

DISCUSSION

It has been repeatedly demonstrated that marked changes in the level of plasma fibrinogen may occur in a variety of circumstances (Homburger, 1945 ; Gilchrist & Tullock, 1952). These changes are, however, slow in onset and may be due to increased synthesis or to shifts in body water. It has been suggested that the level of plasma fibrinogen is dependent, in part, on the level of blood pressure (Sütö-Nagy, 1944). The data obtained in these experiments do not support such an hypothesis in that no changes were obtained with the pressor or depressor agents used. An immediate rise in fibrinogen depends upon at least two prerequisites: fibrinogen must be stored in a labile pool and the drug used must be able to cause its release. It is well known that some anaesthetic agents are capable of depressing a number of physiological responses. However, our use of two different anaesthetic agents and the experiments with unanaesthetized dogs seem to rule out any likelihood that the drugs were prevented from acting by the conditions of the experiments. Furthermore, the chance that the fibrinogen pool had already been depleted by any stress factor as a result of dogs kept in a pound environment is obviated by the employment of animals housed for a time in veterinary quarters. The lower levels of fibrinogen in these latter dogs indicate that the level of circulating fibrinogen is dependent, in part, upon the state of nutrition and environment of the animals.

Because early preparations of adrenaline are known to have been mixtures of adrenaline and noradrenaline, a mixture was used in two experiments, but the results were identical with the previous experiments. Therefore, on the basis of the experiments reported in this paper, we conclude that exogenous adrenaline, noradrenaline, and isopropylnoradrenaline, and thus probably the sympathetic nervous system, are not involved in an immediate increase in plasma fibrinogen as previously reported (Rieker & Winters, 1931 ; Goreczky & Kováts, 1942). Our experiments do not, of course, eliminate the possibility that these catecholamines are involved in a slow fibrinogen response.

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ANTI-TRYPANOSOMAL ACTIVITY OF CERTAIN PHENYLDIAZOAMINO- AND PHENYLAZOAMINO-PHENANTHRIDINIUM COMPOUNDS

BY

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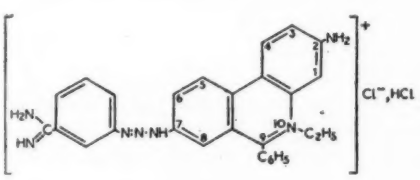
Red phenyldiazoaminophenanthridinium and purple phenylazoaminophenanthridinium compounds, most of which were obtained as isomeric pairs, were tested in mice for curative and prophylactic action against *Trypanosoma congolense*. Several of the individual compounds showed a high degree of activity, the red isomer of each pair usually being more active therapeutically and prophylactically than the purple one, although high prophylactic effect was not always associated with high therapeutic potency. The most interesting isomeric pair was that consisting of metamidium, 7-(*m*-amidinophenyldiazoamino)-2-amino-10-ethyl-9-phenylphenanthridinium chloride hydrochloride (red isomer) and *x*-(*m*-amidinophenylazo)-2,7-diamino-10-ethyl-9-phenylphenanthridinium chloride hydrochloride (purple isomer). Among the individual compounds, the one with the greatest curative action was the red isomer of metamidium, now known as isometamidium. Its 10-methyl homologue was slightly less active and its *p*-amidino-isomer was considerably less active. The compounds with the greatest prophylactic action were 2,7-di(*m*-amidinophenyldiazoamino)-10-ethyl-9-phenylphenanthridinium chloride dihydrochloride trihydrate, its 10-methyl homologue, and the red *m*-amidino-isomers mentioned above. The only other compounds to show appreciable prophylactic action were three guanidino-substituted phenyldiazoaminophenanthridines, although they were less active than metamidium at low doses. One of these, 2-amino-10-ethyl-7-(*m*-guanidinophenyldiazoamino)-9-phenylphenanthridinium chloride hydrochloride, was also very active therapeutically.

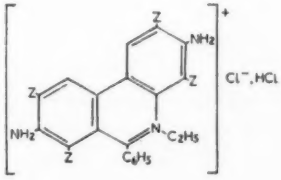
Several phenanthridinum compounds were shown by Browning, Morgan, Robb & Walls (1938), Wien (1946), Walls (1947), Browning (1949), Brownlee, Goss, Goodwin, Woodbine & Walls (1950) and Woolfe (1952, 1956) to possess considerable anti-trypanosomal activity. Of these, 2,7-diamino-10-methyl-9-phenylphenanthridinum bromide (dimidium bromide) and its 10-ethyl analogue (homidium bromide) have been used extensively as curative agents for bovine trypanosomiasis. More recently another phenanthridinium compound, 2-amino-7-(2-amino-6-methylpyrimid-4-ylamino)-9-(*p*-aminophenyl)-10-methylphenanthridine 10,1′-dimethobromide (Prothidium bromide), has been found to have a prophylactic effect superior to that of quinapyramine prophylactic B.Vet.C. (Woolfe & Watkins, 1956; Robson & Milne, 1957; Robson & Hope Cawdery, 1958; Smith, 1959).

In 1958 Wragg, Washbourn, Brown & Hill described the chemical and biological properties of metamidium, prepared by the condensation of *m*-amidinobenzenedia-

zonium chloride and homidium chloride. The product was obtained as a mixture of *red* and *purple* isomers, to which the structures 2-(*m*-amidinophenyldiazoamino)-7-amino-10-ethyl-9-phenylphenanthridinium chloride hydrochloride and 7-(*m*-amidinophenyldiazoamino) - 2 - amino - 10-ethyl-9-phenylphenanthridinium chloride hydrochloride (I) respectively were provisionally assigned. Recent chemical investigations (Berg, 1960) have shown that in fact the *red* isomer is represented by formula (I) and the *purple* isomer by formula (II). It will be noted that (I) contains the diazoamino structure in which the *m*-amidinophenylazo-group is linked to the aromatic amino-group, and (II) contains the amino-azo-system in which the amidinophenylazo-group is coupled directly to the aromatic ring at one of the positions *ortho* to the amino-groups.



I
ISOMETAMIDIUM



II
where one Z = *m*—NH_2·C(:NH)·C_6H_4·N:N—
and the others are H

The numbering of the phenanthridine nucleus in this paper corresponds to the system used in earlier papers on this series of trypanocidal compounds, but differs from the I.U.P.A.C. rules as set out in *Handbook for Chemical Society Authors, 1960*.

The present paper deals with the anti-trypanosomal effect of an analogous series of compounds (Washbourn & Wragg, in preparation ; Berg, in preparation) prepared by the coupling reaction of substituted benzenediazonium chlorides with homidium, dimidium and phenidium salts. Throughout this paper the expression " mixture " refers to the two isomers mixed in the proportions obtained from the coupling reaction between the requisite diazonium and phenanthridinium salts when this reaction is carried out under the conditions described. It does *not* refer to artificially prepared mixtures of the pure isomers.

METHODS

A laboratory strain of *T. congolense* (kindly supplied by Dr W. E. Ormerod), which had been maintained by intraperitoneal syringe-passage in mice over many years, was used in these experiments. It produced a subacute infection resulting in a variable parasitaemia, and the mice normally died within 1 to 3 weeks.

The subcutaneous LD50 of each compound was first determined approximately with 5 mice/dose, and some of the more active compounds were re-examined with 10 mice/dose to obtain a more accurate figure. It was not considered necessary to determine the limits of error of these estimations, and relative toxicities were approximately determined by the relationship of the LD50 figures.

Therapeutic experiments. Mice with an infection in the peripheral blood stream of 1 to 10 trypanosomes/high-power field were given a single subcutaneous injection. Five mice were used for each dose in the preliminary screening tests and 10/dose in the subsequent experiments

with the more active drugs. Wet blood smears were examined 3 times a week for 4 weeks, and the number of animals cleared of trypanosomes during that period (and therefore considered "cured") was noted. The mean curative dose (CD50) was then obtained either by inspection or graphically by de Beer's method (1945), and the approximate relative activities were determined by the relationship of the CD50 figures.

Prophylactic experiments. In these experiments a single subcutaneous injection of the substance was given to a number of mice, and at intervals groups of 6 or more were challenged intraperitoneally with *T. congolense* (approximately 65,000 trypanosomes/mouse). No mouse was challenged more than once. The mice were examined for trypanosomes for 4 weeks after challenge, as in the therapeutic experiments, and those which remained clear of trypanosomes for this period were considered to have been protected. Untreated controls became positive for trypanosomes within 5 days.

In the earlier toxicity, therapeutic and prophylactic experiments, homidium chloride (B.Vet.C.) was used as the reference compound, but later it was replaced by metamidium chloride.

RESULTS

For homidium, the LD50 ranged from 50 to 100 mg/kg and the CD50 from 0.03 to 0.2 mg/kg. To facilitate comparisons of potency of the compounds shown in Tables 2 to 5, their toxicity and activity have been expressed as homidium equivalents (homidium in the same experiment = 1). When metamidium was used as a standard of comparison, potencies were recalculated to give the homidium equivalent on the basis of metamidium being one-third as toxic and four times as active as homidium in a direct comparison (Table 1).

TABLE 1

DIRECT COMPARISON OF THE TOXICITY AND THERAPEUTIC ACTIVITY AGAINST *T. CONGOLENSE* IN MICE OF HOMIDIUM AND METAMIDIUM INJECTED SUBCUTANEOUSLY

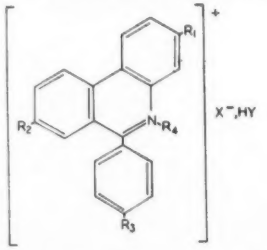
Compound	LD50 mg/kg	Limits of error % (P=0·05)	Slope "b"	CD50 mg/kg	Limits of error % (P=0·05)	Slope "b"
Homidium	75·0	85–115	6·1	0·03	73–137	4·1
Metamidium	210·0	84–119	7·0	0·0075	76–135	4·5

In the prophylactic experiments, except those shown in Table 6, groups of mice were challenged 3, 6, 9 or 12 weeks after dosing with 25 mg/kg. The prophylactic period given in the tables is that for which complete protection was provided. Homidium failed to protect mice after 3 weeks.

m- *and* p-*Amidinophenyl compounds.* All the compounds in this group were less toxic than homidium. This difference was not simply the effect of their generally lower solubility, because those compounds that were relatively soluble were still only about one-third as toxic as the standard compound. The red isomer of each pair was more active than the purple one. Also, greater therapeutic activity resulted from *m*-amidino- than from *p*-amidino-substitution and from 10-ethyl than from 10-methyl quaternation. Maximum activity was found in the red *m*-amidino-10-ethyl isomer (M&B 4180, the bromide of isometamidium) (Table 2), which was about 14 times as active as homidium. M&B 5853 (Table 2), the deamino-analogue

TABLE 2

THE ACTIVITY OF SOME PHENYLDIAZOAMINOPHENANTHRIDINIUM SALTS (*RED* ISOMERS) AGAINST *T. CONGOLENSE* IN MICE



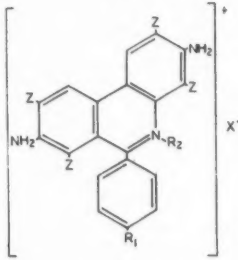
The doses and homidium equivalents are given in terms of the salts (cation content 70–83·5%). M&B 4180 is the bromide of isometamidium. M&B 4616 and M&B 4492 were obtained by the coupling reaction of diazotized 7-acetamido-2-amino-10-methyl (*or* ethyl)-9-phenylphenanthridinium chloride and *m*-aminobenzamidine monohydrochloride. M&B 4474 may equally well have the isomeric structure in which R_2 and R_3 are interchanged. This orientation has not been investigated. M&B 4762 is a mixture of two isomers, one with the structure given below, the other in which R_2 and R_3 are interchanged

(*a*)=*p*-NH_2.C(:NH).C_6H_4.N:N.NH–. (*b*)=CH_3.CO.O–. (*c*)=*m*-NH_2.C(:NH).C_6H_4.N:N.NH–

M&B no.	R_1	R_2	R_3	R_4	X	Y	Solubility w/v water	Relative toxicity (homidium=1)	Relative activity (homidium=1)	Protection (in weeks) afforded by 25 mg/kg subcutaneously
4261	$-NH_2$	*p*-NH_2.C(:NH).C_6H_4.N:N.NH–	H–	C_2H_5–	Cl	Cl	18%	0·3	0·05	<3
4180	$-NH_2$	*m*-NH_2.C(:NH).C_6H_4.N:N.NH–	H–	C_2H_5–	Br	Br	0·7%	<0·25	14·0	>12
5853	–H	*m*-NH_2.C(:NH).C_6H_4.N:N.NH–	H–	C_2H_5–	Cl	Cl	2·0%	0·3	0·7	3
4492	*m*-NH_2.C(:NH).C_6H_4.N:N.NH–	CH_3.CO.NH–	H–	C_2H_5–	Cl	Cl	0·6%	<0·1	0·002	Not tested
4616	*m*-NH_2.C(:NH).C_6H_4.N:N.NH–	CH_3.CO.NH–	–H	CH_3–	Cl	Cl	0·5%	0·1	<0·001	Not tested
4415	$-NH_2$	*m*-NH_2.C(:NH).C_6H_4.N:N.NH–	–H	CH_3–	Br	Br	<0·1%	<0·02	12	>9
4474	–H	$-NH_2$	(*a*)	CH_3–	Cl	(*b*)	<0·1%	<0·1	<0·001	Not tested
4762	–H	$-NH_2$	(*c*)	CH_3–	Cl	Cl	0·3%	1·0	0·005	<3
4814	$-NH_2$	*p*-NH_2.C(:NH).NH.N:CH.C_6H_4.N:N.NH–	–H	C_2H_5–	Cl	Cl	4·0%	0·03	0·02	12
4907	$-NH_2$	*m*-NH_2.C(:NH).NH.N:CH.C_6H_4.N:N.NH–	–H	C_2H_5–	Cl	Cl	2·5%	<0·1	0·01	12
4806	$-NH_2$	*m*-NH_2.C(:NH).NH.C_6H_4.N:N.NH–	–H	C_2H_5–	Cl	Cl	2·0%	0·1	2·0	6

TABLE 3

THE ACTIVITY OF SOME AMIDINOPHENYLAZOPHENANTHRIDINIUM SALTS (*PURPLE* ISOMERS) AGAINST *T. CONGOLENSE* IN MICE



where one Z=amidinophenylazo and the others are H

The doses and homidium equivalents are given in terms of the salts (cation 70–83·5%)

M&B no.	R_1	R_2	Z	X	Solubility w/v water	Relative toxicity (homidium=1)	Relative activity (homidium=1)	Protection (in weeks) afforded by 25 mg/kg subcutaneously
3989	H–	C_2H_5–	*p*-NH_2.C(:NH).C_6H_4.N:N–	Cl	0·3%	0·1	0·025	<3
4250	H–	C_2H_5–	*m*-NH_2.C(:NH).C_6H_4.N:N–	Br	0·7%	0·13	1·0	<4
3813	H–	CH_3–	*p*-NH_2.C(:NH).C_6H_4.N:N–	Cl	1%	0·1	0·01	<3
4418	H–	CH_3–	*m*-NH_2.C(:NH).C_6H_4.N:N–	Cl	>5%	0·25	0·5	<3
4669	NO_2–	CH_3–	*m*-NH_2.C(:NH).C_6H_4.N:N–	Cl	3%	0·3	0·1	3

of the red isomer, was slightly less active than homidium. M&B 4616 and 4492 (Table 2), the 7-acetamido-2-(*m*-amidinophenyldiazoamino)-analogues of dimidium and homidium respectively, like the phenidium derivatives, M&B 4474 and 4762 (Table 2), were very much less active than homidium.

The prophylactic activity of M&B 4634 (Tables 4 and 6) was slightly superior at 25 mg/kg to that of metamidium. In both these mixtures the prophylactic activity was due primarily to the red isomer, the purple one being virtually inactive. Equally active prophylactically was M&B 4596 (Tables 5 and 6), 2,7-di(*m*-amidinophenyldiazoamino)-10-ethyl-9-phenylphenanthridinium chloride dihydrochloride trihydrate, although it was considerably less effective therapeutically (Berg, Brown, Hill & Wragg, 1961). A similar result was obtained with the corresponding 10-methyl derivative (M&B 5657).

TABLE 4

THE ACTIVITY OF M&B 4634 AND METAMIDIUM (MIXED ISOMERS) AGAINST *T. CONGOLENSE* IN MICE

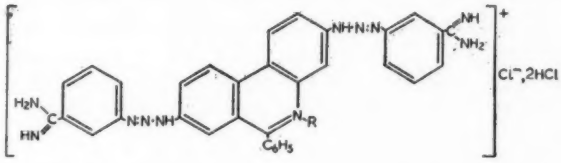
The doses and homidium equivalents are given in terms of the salts (cation contents 75–77·7%). M&B 4180A (isometamidium) and 4250A are the chlorides of M&B 4180 and 4250 respectively. M&B 4634 is the 10-methyl quaternary homologue of metamidium

Compound	Composition	Solubility w/v water	Relative toxicity (homidium=1)	Relative activity (homidium=1)	Protection (in weeks) afforded by 25 mg/kg subcutaneously
M&B 4634	M&B 4415 (Table 1) 40–45% M&B 4418 (Table 2) 55–60%	5%	0·3	2·0	20
Metamidium (M&B 4404)	M&B 4180A 40–45% M&B 4250 50–60%	12·5%	0·3	4·0	16

TABLE 5

THE ACTIVITY OF TWO DI(*m*-AMIDINOPHENYLDIAZOAMINO)PHENANTHRIDINIUM SALTS AGAINST *T. CONGOLENSE* IN MICE

The doses and homidium equivalents are given in terms of the salts (cation contents 70·8–71%)



M&B no.	R	Solubility w/v water	Relative toxicity (homidium=1)	Relative activity (homidium=1)	Protection (in weeks) afforded by 25 mg/kg subcutaneously
5657	Me	2%	<0·05	0·01	12
4596	Et	1·5%	<0·1	0·05	>24

Halogen substitution in the amidinophenyl ring. The introduction of a chlorine atom in the *meta*-position to the amidino-group of metamidium reduced the therapeutic activity somewhat, although the compound still had a better therapeutic

TABLE 6

DIRECT COMPARISON OF THE PROPHYLACTIC ACTIVITY AGAINST *T. CONGOLENSE* IN MICE OF METAMIDIUM WITH M&B 4596, M&B 4634, M&B 4814 AND M&B 4907

The formulae of these compounds are given in Tables 3, 4, 3, 1 and 1 respectively

	Dose injected subcutaneously		No. of mice protected/no. treated. Weeks after dosing										
Compound	mg/kg	mg (cation)/kg	2	3	4	6	8	9	12	16	20	24	26
Metamidium	25	20		6/6		5/5		6/6	11/11	10/10	11/14	8/10	
	5	4			4/4			4/6					
	1	0·8	6/6		19/19	9/14							
M&B 4596	25	18		6/6		5/5		6/6	20/20	19/21	15/15	10/10	
	5	3·6			4/5			4/4					
	1	0·7	5/6		11/19	9/16							
Metamidium	25	20								10/11	6/10		0/5
	5	4			4/4			4/6					
	1	0·8	6/6		5/5			0/3					
M&B 4634	25	19								5/5	12/12		9/10
	5	3·8			6/6			6/6					
	1	0·75	4/4		3/6			0/4					
Metamidium	25	20							6/6	9/10	4/6	0/7	
	5	4			6/6		5/5						
M&B 4814	25	20							5/5	6/8	1/4		
	5	4			0/6		0/8						
M&B 4907	25	20							10/10	8/10	3/3	1/6	
	5	4			0/6		0/8						

ratio than homidium, and decreased prophylactic activity drastically so that the compound failed to protect mice for 3 weeks. The relative proportion of red and purple isomers in this mixture was similar to that in metamidium.

Basic substituents other than an amidino-group. Of three guanidino-substituted compounds (Table 2), M&B 4806, with a 7-*m*-guanidinophenyldiazoamino-substituent, was the most active therapeutically. It was, however, less curative than the corresponding *m*-amidino-compound, M&B 4180, and also less prophylactic. The analogous *m*-guanidinoiminomethylphenyl derivative (M&B 4907, Table 2) had a much lower therapeutic activity but increased prophylactic effect, while the *p*-guanidinoiminomethylphenyl derivative (M&B 4814) was as active but more prophylactic than the *p*-amidino-compound, M&B 4261 (Table 2). M&B 4814 is outstanding in being the only *para*-substituted compound to show any great degree of prophylactic activity.

2,7- Diamino-*x*- (4 - dimethylaminophenylazo) -10-ethyl-9-phenylphenanthridinium methobromide bromide and 2,7-diamino-*x*-(4-diethylaminoethoxycarbonylphenylazo)-10-ethyl-9-phenylphenanthridinium chloride hydrochloride were much less active therapeutically than homidium.

Sulphamoyl, carbamoyl and nitro-substitution. Replacing the *p*-amidino-group in M&B 4261 (Table 2) and in M&B 3989 (Table 3) by a sulphamoyl or carbamoyl group had little influence on therapeutic activity; replacing it by a chlorine atom or by a carboxyl group reduced the therapeutic activity. Replacement of the *m*-amidino-group in the isomers of metamidium by a sulphamoyl or carbamoyl group resulted in a marked reduction of therapeutic activity to a value below that

for the corresponding *para*-substituted compounds. This applied also to the prophylactic activity of the red *m*-sulphamoyl isomer. Replacement of the *m*-amidino-group in the metamidium isomers by a nitro-group also reduced therapeutic activity. 2-Amino-7-(*o*-chlorophenyldiazoamino)-10-ethyl-9-phenylphenanthridinium chloride and the corresponding purple isomer were less active than homidium.

DISCUSSION

The general conclusions which may be drawn from this work are (*a*) that an amidino-group was superior to any of the other substituents which were tried, (*b*) that *meta*-substitution was better than *para*-substitution, and (*c*) that the red phenyldiazoamino-isomers were more active than the purple phenylazoamino- ones.

Of all the compounds tested, seven combined some curative action with a considerable degree of prophylactic activity. They were the bromide of isometamidium (M&B 4180) and its methyl quaternary homologue (M&B 4415), the 2,7-disubstituted analogue of metamidium (M&B 4596) and its methyl quaternary homologue (M&B 5657), and three guanidines listed in Table 2. Of these seven, the first pair were outstanding, and mixed with their corresponding purple isomers, to give metamidium and M&B 4634 respectively, they maintained a high curative and prophylactic potency.

Comparative tests showed that a subcutaneous dose of 25 mg/kg of M&B 4634 gave more prolonged protection that the same dose of metamidium, in spite of the fact that M&B 4634 was slightly less active therapeutically than metamidium. With M&B 4596, 4814 and 4907 the contrast was even greater since they were no less protective than metamidium at 25 mg/kg but were very much less active therapeutically. Because of this prolonged protection they must be intrinsically very active compounds. A possible explanation of their poor therapeutic effect is that they are fixed in the host's tissues more rapidly than metamidium, before their full anti-trypanosomal effect is manifested.

Isometamidium (M&B 4180A) and M&B 4596 are now under test in African cattle. The results so far are very encouraging. The toxicity and activity in African cattle of metamidium (M&B 4404) have been reported by Fairclough (1958), Whiteside (1958, 1960), Fiennes (1960), Kirkby (1960), Smith & Brown (1960), Stephen (1960) and others. The local tolerance of cattle to subcutaneous injections is poor; intramuscular injections are better tolerated, however, and satisfactory results have been recorded in most instances where doses of 3 mg/kg or less have been used. An exception exists in the case of Northern Nigerian cattle, which often react severely to relatively small doses. Systemic tolerance is generally satisfactory, provided a dose of 3 mg/kg is not exceeded, although here again exceptions have been recorded, notably amongst Nigerian Fulani cattle. The reasons for the exceptionally poor tolerance of Northern Nigerian cattle to metamidium and to certain other phenanthridines are not understood.

Experiments in East and West Africa showed metamidium to be considerably more active curatively against *T. congolense* than homidium, and data from trials conducted in several localities suggest that a dose of 0.5 to 1.0 mg/kg may be relied upon to eliminate most of the trypanosomes encountered under field conditions.

In a heavy challenge (*T. congolense, T. vivax* and *T. brucei*) in East Africa an intramuscular dose of 4 mg/kg protected cattle for an average of 18 weeks, compared with 10 weeks for 11.7 mg/kg of quinapyramine (prophylactic) in the same trial (Smith & Brown, 1960). In West Africa one of seven Fulani cattle given 3 mg/kg succumbed to a natural challenge (*T. congolense* and *T. vivax*) after 15 weeks (Kirkby, 1960).

In laboratory tests with cattle (Whiteside, 1960), metamidium has been found to cure strains of *T. congolense* resistant to quinapyramine, homidium, dimidium, Prothidium and Berenil, and therefore has great value as a "sanative." Strains resistant to metamidium could be cured satisfactorily with Berenil.

We are very grateful to our colleagues, Mr S. S. Berg, Mr L. Bretherick, Mr K. Washbourn and Dr W. R. Wragg for supplying the compounds, and to Mr C. G. L. Beveridge for compiling the summary of the field trials with metamidium. We also wish to thank our colleagues, Dr J. N. Ashley, Dr H. J. Barber and Dr R. Wien, for their helpful discussions, and Miss G. Merchant and Mr T. Mercer for technical assistance.

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5-HYDROXYTRYPTAMINE AND TRANSMISSION IN SYMPATHETIC GANGLIA

BY

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Rat stellate ganglia were isolated and maintained in a moist chamber in which they were intermittently immersed in physiological saline containing low concentrations of the compound under investigation. 5-Hydroxytryptamine (serotonin) in concentrations as low as 1.3×10^{-6} M reduced reversibly the threshold and increased the amplitude of responses to preganglionic stimulation. Certain antagonists of 5-hydroxytryptamine depressed reversibly the postsynaptic responses. Experiments *in vivo* supported this finding. It is suggested that 5-hydroxytryptamine facilitates transmission in the sympathetic ganglia of rats.

The effect of 5-hydroxytryptamine on transmission of nerve impulses in the autonomic nervous system has been explored by several investigators. Marrazzi & Hart (1953) reported that, while 65 μg/kg of 5-hydroxytryptamine had no effect on nonsynaptic potentials, it blocked reversibly ganglionic transmission in C fibres of the ciliary ganglion preparation of the dog. Douglas & Ritchie (1957) observed a decline in potentials evoked antidromically in C fibres of the vagus nerve of rabbits after intravenous injection of 5-hydroxytryptamine (10 to 100 μg) and concluded that spontaneous activity of the fibres was thereby increased. Marrazzi (1957) reported that 5-hydroxytryptamine induced synaptic inhibition in transcallosal pathways in the cat (10 μg/kg).

Trendelenburg (1957) showed that intra-arterial injections of 5-hydroxytryptamine potentiated the response of the cat nictitating membrane to submaximal stimulation of the cervical sympathetic trunk and concluded that this was due to an action of the compound upon the ganglion cells although the possibility of an effect on the end organ could not be excluded. Bindler & Gyermek (1961) found that 2.5 to 15 μg of 5-hydroxytryptamine by intra-arterial injection increased spontaneous activity in the postganglionic fibres from the inferior mesenteric ganglion.

The occurrence of 5-hydroxytryptamine in vertebrate sympathetic ganglia has, as yet, not been demonstrated. However, Gertner, Paasonen & Giarman (1957) observed that the cat superior cervical ganglion is capable of forming 5-hydroxytryptamine when perfused with iproniazid but found that this monoamine oxidase inhibitor produced partial inhibition of transmission through the ganglion. They

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stated that homogenates of sympathetic ganglia possess a higher capacity for catalytic formation of 5-hydroxytryptamine than did any other structure of the nervous system.

The following experiments were performed in an attempt to shed some light on the role of 5-hydroxytryptamine in sympathetic activity.

A preliminary report of this work was made to the meeting of the Federation of American Societies for Experimental Biology in 1960 at Atlantic City, N.J., U.S.A.

METHOD

Preparation of tissue

A portion of the thoracic and cervical regions of the sympathetic trunk was rapidly dissected free from adult Sprague-Dawley rats. The tissue isolated consisted of the stellate ganglion, about 5 mm of the thoracic chain and the entire cervical sympathetic trunk exclusive of the superior cervical ganglion. It was essential that the sheath or capsule surrounding the stellate ganglion be partially or completely removed during the dissection. The preparation was bathed in Krebs solution with a reduced bicarbonate concentration as recommended by Eccles (1952). The solution was equilibrated with 95% oxygen and 5% carbon dioxide to a *p*H of 7.3 to 7.4 at 35 to 36° C.

Apparatus

The preparation was suspended horizontally on platinum electrodes as shown in Fig. 1. The thoracic or preganglionic end was stimulated with rectangular pulses of 0.1 msec duration and an intensity of 0.1 to 15 volts. In experiments on the amplitude of responses, a stimulus intensity was chosen which elicited about one-third of the maximal response. This submaximal stimulus intensity, once chosen, remained unchanged during the experiment.

The threshold of responses was determined by varying the intensity of the preganglionic stimulus to obtain a constant but minimal response.

The problem of maintaining the tissue in a liquid environment but recording in a non-conducting medium was met by automatically lifting the preparation out of solution periodically (Fig. 2). A programme timer controlled a solenoid which, when activated, pulled the electrode assembly downward and hence immersed the ganglion preparation in Krebs or test solution.

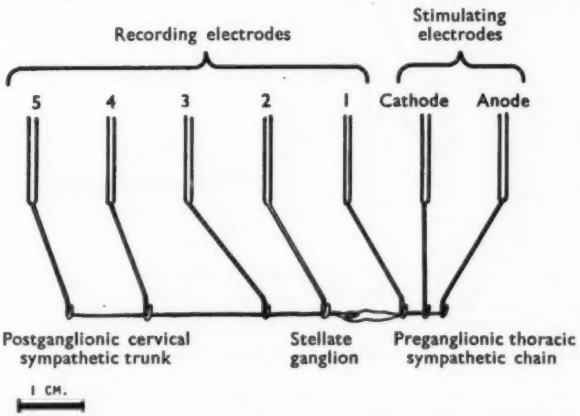

Fig. 1. Arrangement of sympathetic trunk on electrodes. Stimulating pulses were applied through a radio-frequency isolation unit. Recording electrode 1 was earthed and records were obtained from electrodes 1 and 2, or 1 and 3, 4 or 5.

The immersion continued for 30 sec of each minute after which the solenoid circuit was broken and spring tension pulled the electrode carrier out of the solution into the moist air of the chamber. This chamber was virtually sealed and its water jacket held the temperature at 36° C. Stimulation and recording were automatically performed after 25 sec of equilibration in the moist air and 5 sec later the immersion was repeated. This cycle was continued throughout the experiment.

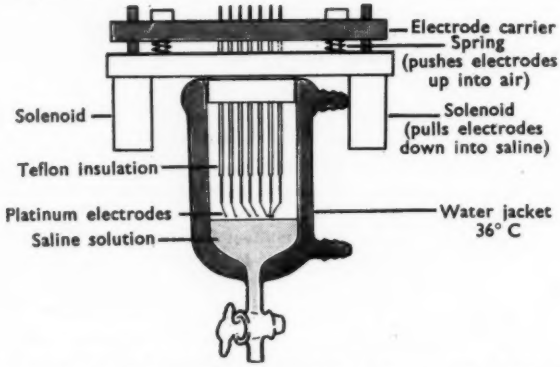

Fig. 2. Details of construction of nerve chamber and electrode assembly. Nerve was periodically immersed in solution and lifted out for recording by the action of a solenoid controlled by a programme timer.

Attempts to perform the sequence of events of immersion, elevation, equilibration with moist air and stimulation manually indicated that timing of the events must be precise if reproducible recordings were to be obtained.

All experiments were performed during the first few hours after dissection although the preparation was responsive for many hours. The potential S_b (Fig. 3) could be elicited for 6 or 8 hr while potential S_a could be elicited for 72 hr.

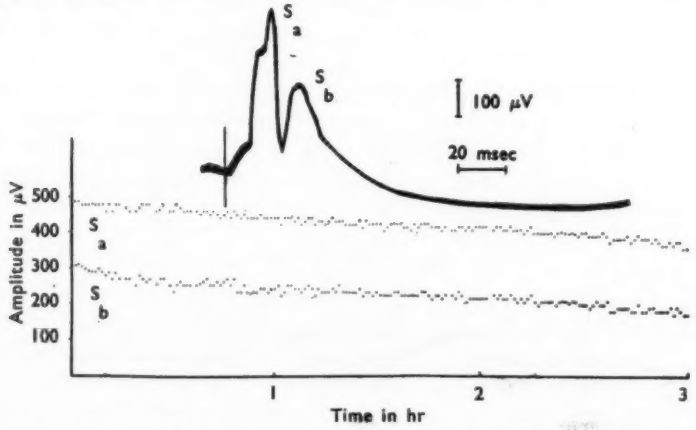

Fig. 3. Above: Response to a submaximal preganglionic stimulus. Records obtained between electrodes 1 and 2 of Fig. 1. Intermittent exposure to control solution only. Below: Scatter diagram of amplitudes of responses (S_a and S_b) at 1 min intervals over a 3-hr period.

Experiments in vivo

Three experiments were performed in which rats under light pentobarbitone anaesthesia were artificially respired. Stimulating electrodes were placed on the thoracic sympathetic trunk and recording electrodes on the ipsilateral cervical sympathetic trunk, thus simulating the experimental conditions used with the isolated ganglion.

RESULTS

Fig. 3 (upper part) illustrates the response obtained from a single submaximal preganglionic stimulus. The artifact is followed by a complex of responses. In conformity with previously accepted terminology, the major components are labelled S_a and S_b. This paper is concerned with the effects of compounds on the amplitudes of these two responses or on the stimulus thresholds necessary to arouse these responses.

Fig. 3 (lower part) gives a scatter diagram of the amplitudes of responses obtained at 1 min intervals for 3 hr during intermittent immersion in Krebs solution. There is shown a slow decline in responses at the rate of about 0.25%/min and a maximum variation or scatter of 10% from an eye fit line of regression.

In preliminary work, 112 experiments were made before the experimental method was developed in its final form. Then 40 measurements were made with various concentrations of 5-hydroxytryptamine. The amplitude of the response was increased by 5-hydroxytryptamine in 36 experiments (range +7.4 to +206%); there was no change in 3 and there was a decrease in 1 (−4%).

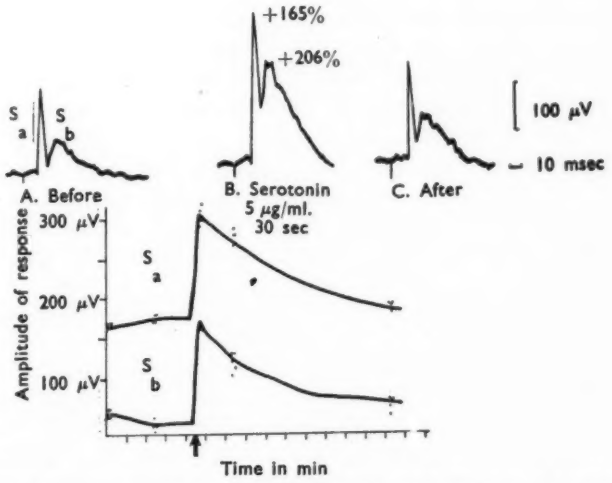

Fig. 4. Above: Responses recorded between electrodes 1 and 2 of Fig. 1. A, Control, response before experiment; B, experimental, response immediately after exposure of preparation to 5-hydroxytryptamine for 30 sec; C, recovery, response after 10 min in control solution (Krebs). Below: Time course of amplitude of S_a and S_b responses. Arrow indicates time of immersion in 5-hydroxytryptamine for 30 sec.

The quantitative variation seen in these results may reflect uncontrolled variables such as injury to the tissue, variable degree of penetration of 5-hydroxytryptamine into the active cells and the impossibility of using comparable submaximal stimuli in successive preparations.

After exposure for 30 sec to 5 μg/ml. (1.3×10^{-5} M) of 5-hydroxytryptamine creatinine sulphate added to the chamber fluid, there was an immediate effect on the response amplitude (Fig. 4). The amplitude of S_a had increased to 165% and that of S_b had increased to 206% of the control responses. Washing in Krebs solution for about 20 min was necessary before the potentials returned to their original values.

TABLE 1

EFFECT OF 5-HYDROXYTRYPTAMINE ON AMPLITUDE ON THE S_a AND S_b RESPONSES

Conc. of 5-hydroxytryptamine (μg/ml.)	Change in amplitude (%)	
	S_a	S_b
0·5	14	31
2·0	20	48
4·0	23	52
8·0	40	86
16·0	40	94
32·0	33	76
64·0	30	27

The amplitude of the response was related to the concentration of 5-hydroxytryptamine. In the experiment in Table 1, the maximum increase in response was produced by a 5-hydroxytryptamine concentration of 16 μg/ml. (4.2×10^{-5} M), while higher concentrations caused less increase.

These results suggested that 5-hydroxytryptamine might be acting by reducing the threshold of preganglionic fibres since submaximal stimuli were used. With a lower threshold, a greater number of fibres would be activated by a given submaximal stimulus and this change in threshold might account for the increased amplitudes of both responses.

Determination of the effect on threshold proved difficult. In most experiments, the threshold of responses to preganglionic stimulation was not sharply limited. However, in some ganglia this threshold was especially critical and the value of minimal stimulus intensity for a constant, small response did not vary during

TABLE 2

EFFECT OF 5-HYDROXYTRYPTAMINE ON THRESHOLD OF RESPONSES

Conc. of 5-hydroxytryptamine (μg/ml.)	Threshold change (%)
0·1	−5
0·5	−11
5·0	−23
40·0	+18

repeated trials. In such a preparation low concentrations of 5-hydroxytryptamine reduced the threshold of responses to preganglionic stimuli whereas high concentrations had the opposite effect (Table 2). In most experiments, S_b response was more sensitive to the addition of 5-hydroxytryptamine than was the S_a response.

A series of ganglion blocking agents was next used to find whether they, too, had a differential effect upon the two potentials. Tetraethylammonium, tubocurarine and pempidine all had a greater effect on S_b than on S_a (Table 3).

TABLE 3

EFFECT OF GANGLION BLOCKING AGENTS ON AMPLITUDE OF RESPONSES

		Change in amplitude (%)	
Agent	Conc.	S_a	S_b
TEAC	5 μg/ml.	−10	−36
	10	−25	−75
	250	−100	−100
Tubocurarine	0·3 mg/ml.	Unchanged	Unchanged
	0·6	Unchanged	Unchanged
	0·9	−10	−22
	1·2	−10	−50
	1·5	−20	−55
	3·0	−20	−100
Pempidine	1 μg/ml.	−2	−33
	6	−18	−61
	10	−41	−84

TEAC=Tetraethylammonium chloride (Etamon chloride), Parke-Davis. Tubocurarine=Intocostrin, tubocurarine chloride pentahydrate, Squibb. Pempidine tartrate, May and Baker.

Similar experiments were performed using some "anti-serotonin" substances. Bromolysergic acid diethylamide increased the amplitude of responses in low concentration but decreased them in higher concentration (Table 4). Methysergide (1-methyl-D-lysergic acid butanolamide) had no effect in low concentrations and increased responses at higher concentrations.

TABLE 4

EFFECT OF ANTI-5-HYDROXYTRYPTAMINE SUBSTANCES ON THE AMPLITUDE OF RESPONSES

		Change in amplitude (%)	
Agent	Conc.	S_a	S_b
BOL-148	2 μg/ml.	+28	+17
	5	−22	−33
UML-491	2 μg/ml.	Unchanged	Unchanged
	5	Unchanged	Unchanged
	15	+15	+18
	25	+15	+32

BOL-148=Bromolysergic acid diethylamide bitartrate, Sandoz.
UML-491=Methysergide, Sandoz.

It was of interest to know whether similar results could be obtained in the intact animal. Rats, lightly anaesthetized with pentobarbitone, were artificially respired while the chest was opened and stimulating electrodes were placed on the sympathetic trunk at the level of the third to fourth rib. Polyethylene plastic film was used to prevent the electrodes from coming in contact with nearby tissues and the exposed area was covered with medicinal liquid paraffin. The recording electrodes on the cervical sympathetic trunk were also similarly protected. The position of the stimulating and recording electrodes simulated those in the *in vitro* experiments as closely as possible.

The control postganglionic response to a standard submaximal preganglionic stimulus following the intravenous injection of Krebs solution is shown in Fig. 5 (left-hand response). After a single rapid intravenous injection of 20 μg of 5-hydroxytryptamine, the response was increased by 43% in 30 sec (Fig. 5, middle record). An intravenous dose of 5 mg of tetraethylammonium chloride reduced the response by 76% (Fig. 5, right-hand record). In all such experiments the results were similar.

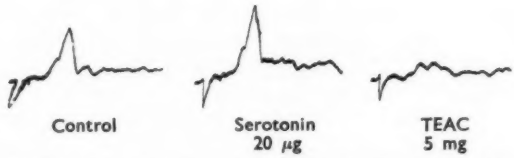

Fig. 5. Records of postganglionic responses to submaximal preganglionic stimuli in the living rat. Left, control; middle, 30 sec after intravenous injection of 20 μg of 5-hydroxytryptamine (serotonin); right, after intravenous injection of 5 mg of tetraethylammonium chloride.

DISCUSSION

The results suggest that low concentrations of 5-hydroxytryptamine increased the electrical response of the rat stellate ganglion evoked by submaximal preganglionic stimulation.

The action potential seen was a compound response with two spikes similar to those designated as S_a and S_b by Eccles (1952). She concluded that both of the components were ganglionic (postsynaptic) in origin; that the "... ganglionic S_a spike discharge begins so soon after the arrival of the fast preganglionic spike that it obscures most of the diphasic preganglionic spike ..." The activity of non-synaptic fibres (those passing through the stellate ganglion to synapse in the superior cervical ganglion) must likewise be masked in the S_a wave form.

Shaw, MacCullum, Dewhurst & Mainland (1951) have also advanced evidence to support the idea that drugs can act differently upon the S_a and S_b response, and contend that there may be two pharmacologically distinct groups of cells in sympathetic ganglia. It is possible that the S_a and S_b components of the action potential in these experiments also represent the activity of two such groups of cells, 5-hydroxytryptamine having a more pronounced effect on the cells responsible for the S_b discharge.

Woolley (1958) has suggested that 5-hydroxytryptamine acts by carrying calcium ions into the uterine muscle cell and thus activates the actomyosin-adenosine triphosphate system. A few experiments were performed in which the technique of Woolley (1958) was adapted to the study of sympathetic ganglia and the results were inconclusive. It did not appear that calcium transport is dependent on 5-hydroxytryptamine in ganglia.

The recent paper by Bülbring & Burnstock (1960) shows that 5-hydroxytryptamine produces a partial depolarization of smooth muscle fibres. If this should also be true for sympathetic nerve fibres, then it would provide an explanation of the changes observed.

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EXCRETION AND RE-EXCRETION OF A BROAD-SPECTRUM PENICILLIN IN BILE

BY

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A technique is described for estimating the biliary excretion, reabsorption and re-excretion of an antibacterial drug in rats. D(−)-6-(α-Amino-α-phenylacetamido)-penicillanic acid (BRL 1341) is rapidly excreted and re-excreted in the bile, in concentrations bactericidal to Salmonellae and other organisms which are usually refractory to antibacterial drugs in this situation. Recoveries of the drug accounted for 20 to 30% of oral or parenteral doses; much of the remainder is probably destroyed in the gut. Results from one patient indicate that D(−)-6-(α-amino-α-phenylacetamido)-penicillanic acid is excreted in high concentration also in human bile.

It is known that a few drugs, such as penicillin G and phenolphthalein, are rapidly excreted in bile. Reabsorption and re-excretion can obviously th n occur, but there is very little quantitative data about this. In the case of antibacterial drugs, the amount of drug distributed in this way, and the relative concentrations thus obtained in the biliary tract and at different levels in the intestine, are obviously important in the therapy of alimentary infection.

We have recently drawn attention to the behaviour of methicillin in this respect (Harrison, White & Stewart, 1960). In the present paper we describe a technique designed to provide quantitative data about reabsorption and re-excretion, particularly with regard to D(−)-6-(α-amino-α-phenylacetamido)penicillanic acid (BRL 1341), another derivative of 6-aminopenicillanic acid which has wider antibacterial activity and may therefore be of use in the treatment of biliary and intestinal infections.

METHODS

White rats, bred in these laboratories, were selected from both sexes, weighing 250 to 300 g. They were anaesthetized with ether and incised 1.0 to 1.5 cm paramedially through the right side of the shaved abdominal wall. The loop of the duodenum and the bile duct were pulled through the incision. A soft polythene cannula (0.5 mm bore × 0.2 mm wall) was introduced through a lumbar-puncture needle into the abdomen and then inserted upwards into the bile duct. This cannula was secured by ligatures around the bile duct and at the point of egress on the abdominal skin. The wound was then closed in layers by cotton sutures. Rats thus treated served as donors of bile, samples being normally collected in 1-hr fractions for 4 to 7 hr, then overnight or longer. The rats were given 0.9% sodium chloride solution *ad lib.* to counteract loss of water and sodium, and light sedation with nembutal overnight. They were kept during this period in cages of soft wire which lightly restrained their movements. The flow of bile under these conditions was 0.5 to 1.2 ml. per hr.

Rats chosen as recipients were operated upon similarly and cannulated. The free end of the biliary cannula from a donor rat was then inserted through a needle-puncture into the duodenum of a recipient rat, immediately proximal to the ampulla of the bile duct. This cannula was fed into the duodenum for 1 to 2 cm and secured at the point of insertion by a purse-string suture through the muscular layer of the duodenal wall. The abdominal incision was then closed around the duodenal tube; donors and recipients were kept alongside each other in restraining cages.

Doses of drugs were then given, orally or parenterally, to the donor rats. Excretion was assessed by collecting and assaying the bile from a control group of cannulated donors; re-excretion was assessed by collecting and assaying the bile from recipient rats. Tail-blood was also collected at intervals from rats in both groups. Separate controls, kept in metabolism cages, were used for assays on urine and faeces, for it was found that laparotomy often caused a delay of 3 to 4 hr in the voiding of urine. D(−)-6-(α-Amino-α-phenylacetamido)-penicillanic acid was given orally via a stomach tube, or intramuscularly, in doses of 100 mg/kg to the donor rats.

The microbiological and chromatographic techniques employed in these studies have already been described (Stewart, 1960; Harrison, White & Stewart, 1960).

RESULTS

The results obtained in typical experiments are shown in Fig. 1. An oral dose of D(−)-6-(α-amino-α-phenylacetamido)penicillanic acid began to be excreted in the bile, in concentrations of 20 to 160 μg/ml., 1 hr after dosing. This concentration increased sharply during the next 2 hr, and peak values of 160 to 440 μg/ml. were obtained at 2.5 to 7.5 hr with considerable variation, in this respect, between individual rats. Plasma levels during this period showed peak values of 8.5 to 11.5 μg/ml., 1 to 3 hr after dosing, falling to 1.0 μg/ml. or less at 4 to 5 hr.

The re-excreted drug, assayed in the bile of the recipient rats, showed much lower peak values of 6 to 30 μg/ml. with a gradual rise spread over 3.5 to 15 hr after the time of administration of the drug to the donor rats. Plasma levels (0.3 to 4.3 μg/ml.) were also lower, though not proportionately. Assays in parallel showed

TABLE 1

EXCRETION (MG IN 24 HR) IN RATS WEIGHING 250 TO 300 G

Excretion of D(−)-6-α-amino-α-phenylacetamido)penicillanic acid in bile, urine and faeces of rats, and re-excretion in bile after 100 mg/kg orally

	Donor rats			Recipient rats.
Rat	Bile	Urine	Faeces	Bile
1	0·711	2·686	0·086	0·12
2	0·873	2·137	0·080	0·20
3	2·225	2·172	0·250	0·50
4	1·853			0·27
5	0·905			0·58
Mean	1·313	2·331	0·135	0·33
% dose	2·8–7·8	8–11	0·3–0·9	35–66

that the donor controls excreted on average about 5% of the dose in the bile in 24 hr (Table 1). The recipients re-excreted 20 to 60% of this quantity. During the same period about 10% of the dose was excreted in the urine and less than 1% in the faeces.

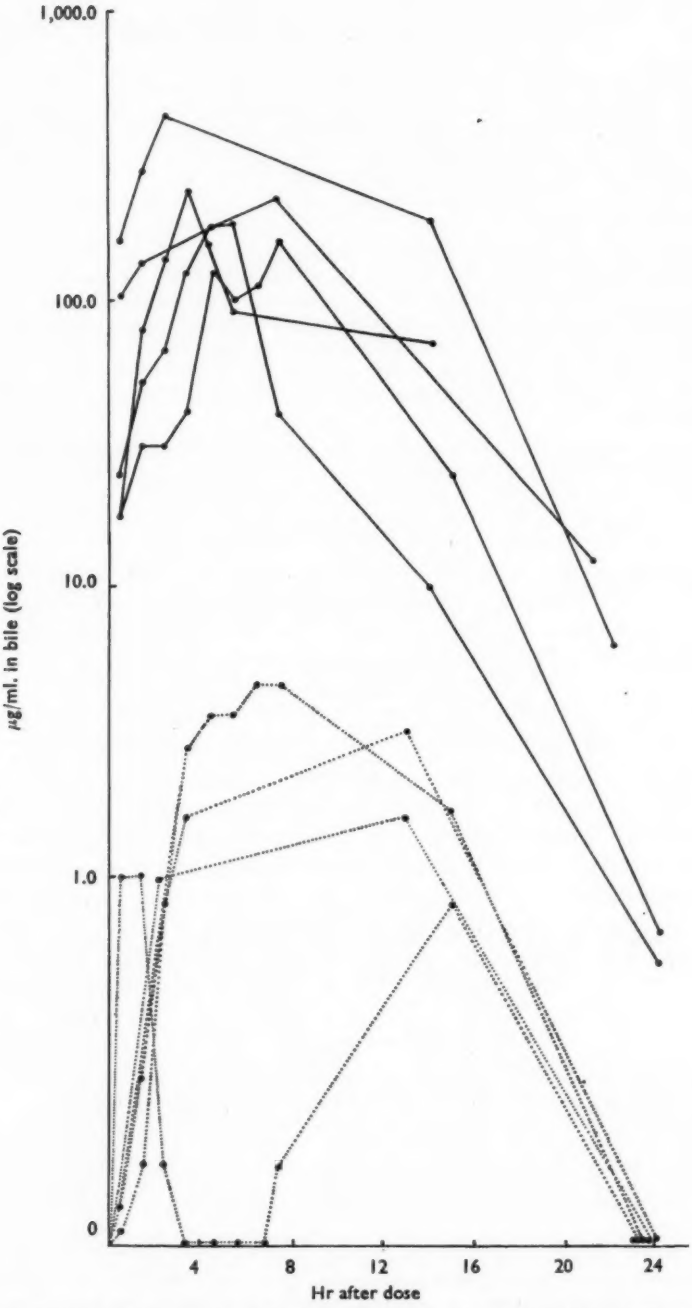

Fig. 1. Excretion and re-excretion of D(−)-6-(α-amino-α-phenylacetamido)penicillanic acid in bile of rats given 100 mg/kg. Donor rats ● —— ●; recipient rats ● · · · · ●.

To check the time factor in absorption from the upper part of the small intestine, the drug was injected directly into the duodenum in two rats. Plasma levels of 1.6 and 3.1 μg/ml. were detected in 15 min. In samples of bile collected during the first hour the concentrations were 114 and 160 μg/ml. Samples taken during the second hour gave values of 126 and 160 μg/ml., declining in succeeding hours. These results indicated that absorption proceeded immediately the drug entered the duodenum and that biliary excretion reached a maximum within 2 hr of this time.

This experiment gave an indication of the rate at which the drug entered the blood stream, but did not exclude absorption into the lymph. To investigate this, 2 rats had cannulae inserted directly into the thoracic duct. Lymph was collected for 3 hr after the standard oral dose and gave on assay only trace concentrations (<1.0 μg/ml.), that is, much lower than the corresponding plasma levels.

The experiments described above provided data from which the absorption and excretion of an oral dose could be calculated, but the actual rate of clearance through the liver could not be estimated accurately owing to the obvious variation in the rate of absorption from the gut. This point was investigated by injecting the drug intramuscularly and assaying samples of bile collected hourly. Under these conditions (Table 2) it was found that very high concentrations (718 to 1,575 μg/ml.)

TABLE 2

EXCRETION OF D(−)-6-(α-AMINO-α-PHENYLACETAMIDO)PENICILLANIC ACID (BRL 1341) IN BILE OF RATS AFTER INTRAMUSCULAR INJECTION (100 MG/KG)

	BRL 1341 in bile		
Hr after injection	Concentration (μg/ml.)	Excretion (μg)	% of dose excreted
1	1,225	980	3·4
	1,545	1,236	4·0
2	1,113	890	3·3
	1,575	2,200	7·3
3	718	790	2·6
	1,250	1,875	6·3
18	40	580	2·0
	20	263	1·0
Total			10·6–18·4

were excreted during the first 3 hr, accounting for a mean excretion of about 5% of the dose per hour. After 3 hr the rate of excretion fell rapidly, the cumulative clearance at the end of 18 hr being 11 to 18% of the dose.

In the evaluation of an antibacterial drug like D(−)-6-(α-amino-α-phenylacetamido)penicillanic acid the concentrations quoted in bile and other body fluids are of therapeutic significance only in so far as they equate to the bacteriostatic or bactericidal levels effective against organisms likely to be carried, or to cause infection, in the biliary tract and upper reaches of the small intestine. The relevant figures for a range of appropriate organisms are shown in Table 3.

Samples of bile and urine were examined chromatographically on Whatman no. 1 paper, by the technique previously described (Stewart, 1960), to see if the excreted drug was the original substance or a metabolite. The dried chromatograms were

TABLE 3

BACTERICIDAL CONCENTRATIONS OF D(−)-6-(α-AMINO-α-PHENYLACETAMIDO)-PENICILLANIC ACID

Organism	No. of strains	No. sensitive	Bactericidal concentration (μg/ml.)
Staph. aureus	282	216	0·1–5·0
Streptococcus			
Group A	57	57	0·05–0·1
Group D	24	22	1·0–5·0
Others	19	19	0·1–5·0
Bact. coli	102	48	1·0–5·0
Salmonella typhi	2	2	2·5
Salmonella typhi-murium	8	8	1·0–5·0
Salmonella, others	5	5	1·0–5·0

cut into strips and laid on nutrient agar already seeded with penicillin-sensitive *Sarcina lutea*. After overnight incubation at 37° C, the R_F values of the samples were calculated by measuring the distances of the solvent front and the zones of inhibition of the test organism from the origin. These measurements showed no difference in the R_F values of the excreted drug and of comparable amounts of pure drug added to bile and urine from control rats.

Since this paper was written, we have assayed D(−)-6-(α-amino-α-phenylacetamido)penicillanic acid in human bile, aspirated from the duodenum of a patient 5 hr after an oral dose, in capsules, of 50 mg/kg. Saline, 20 ml., was injected through the tube before aspiration so that the aspirated bile was diluted. On the assumption that the dilution factor was 1:10 (a minimal estimate) the assay value of the drug in the bile was 22.0 μg/ml. Plasma concentrations were 7.5 μg/ml. at 2 hr and 10 μg/ml. at 6 hr. These results suggest that absorption and biliary excretion proceed on similar lines in humans and rats.

DISCUSSION

These results show that D(−)-6-(α-amino-α-phenylacetamido)penicillanic acid is rapidly absorbed in the upper part of the small intestine in rats and is immediately excreted in active form in the bile. The liver is capable of clearing up to 7% of a large dose from the blood stream in 1 hr, producing a concentration in the bile which may be 40 times higher than the peak plasma concentration and 100 times higher than the concentration required to kill moderately sensitive organisms, such as *Salmonellae* and certain other coliforms; this concentration may actually be 1,000 times that of the level ordinarily effective against many pyogenic cocci.

The technique described enables an assessment to be made of the reabsorption and re-excretion of the drug originally cleared in the bile. Reabsorption produces plasma increments of about 1.0 μg/ml., occasionally more. Hepatic clearance of this amount of drug produces levels in the bile up to about 30 μg/ml., still sufficiently high to inhibit most pathogens. The first phase of absorption of the orally administered drug produces peak plasma levels, and peak excretion, between 1 and 3 hr; the second phase adds small increments to the plasma during this time and up to 6 hr.

In our rats the biliary flow as measured in the cannulated samples was 0.5 to 1.2 ml./hr, which gave an excretion in the post-absorptive stage of up to 0.5 mg of drug per hour. The original oral dose varied, according to body weight, from 25 to 30 mg, and the mean excretion of this, in 24 hr, was 1.3 mg, the greater part having re-entered the gut in the first 3 hr. Simultaneously, about 2.3 mg was excreted in the urine and a trace (0.13 mg) in the faeces. These figures suggest, therefore, that about 1/15th of the original oral dose is immediately excreted in the bile and presumably reabsorbed; during the next 20 hr about 1/5th of this is re-excreted in the bile. Urinary and faecal excretion during this period accounts for about 10% of the dose, so that, even when allowance is made for the fraction undergoing reabsorption and re-excretion, and for the inaccuracies of bioassay, a considerable proportion of the dose is not accounted for. This could mean either that there is retention in the body, or that the greater part of the dose is metabolized. The former possibility—retention—is unlikely, as blood and urine levels fall rapidly after 5 to 7 hr. Metabolism in the body is also unlikely on this scale, as the much higher blood levels produced by parenteral administration are cleared with greater rapidity via the liver and bile. There remains the possibility that the drug is partly destroyed in the gut, and this explanation is favoured by the apparent incompleteness of absorption and reabsorption. The comparative stability of the drug to *p*H and other changes (Rolinson & Stevens, 1961) makes it unlikely that it is destroyed by secretions; but it is known that D(−)-6-(α-amino-α-phenylacetamido)penicillanic acid is susceptible to the penicillin-destroying factors in coliform and other bacteria (Rolinson & Stevens, 1961; Stewart, Coles, Nixon & Holt, 1961) whose presence in the intestine may therefore account for considerable inactivation.

If D(−)-6-(α-amino-α-phenylacetamido)penicillanic acid behaves pharmacologically in man as in the rat, it should have interesting chemotherapeutic possibilities: in therapeutic doses it can attain and maintain in the biliary tract and upper intestine concentrations which are bactericidal to a wide range of organisms, including *Salmonellae* and other pathogens which are often located in these sites; the plasma levels attained by oral dosage indicate that a bactericidal concentration can probably reach many tissues; the urinary concentration is extremely high; and, in all these situations, effective concentrations of active drug are detectable for several hours after a single oral dose. The cycle of absorption-excretion-reabsorption *et seq.* in the gut, together with the possibility of some local destruction of the drug, suggests that it may not reach the ileum in a concentration inhibitory to pathogenic bacteria there. This may explain its failure to clear intestinal carriers of *S. typhi-murium* (Stewart *et al.*, 1961).

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EXCRETION OF ANTIBIOTICS IN BILE

BY

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The excretion of antibiotics in the bile of rats has been studied. Penicillins, including derivatives of 6-aminopenicillanic acid, are rapidly excreted, reabsorbed and re-excreted, in high concentration, whereas streptomycin, neomycin, paramomycin and chloramphenicol reach lower levels in the bile than in the plasma. p-Aminobenzylpenicillin and D(−)-6-(α-amino-α-phenylacetamido)penicillanic acid, both of which are bactericidal to Salmonellae and other coliforms, produce higher concentrations in the bile than benzylpenicillin (penicillin G). This may be of therapeutic importance.

In the preceding paper (Stewart & Harrison, 1961) we drew attention to the high concentration in which D(−)-6-(α-amino-α-phenylacetamido)penicillanic acid was excreted in the bile, and re-excreted after reabsorption. The penicillin family of drugs differs in this respect from the major antibiotics. Since this difference may be of importance in the therapy of infections of the biliary tract and intestine, we report here the results obtained with a range of antibiotics usually considered eligible for the therapy of such infections.

METHODS

The techniques employed in this study are described in the preceding paper (Stewart & Harrison, 1961). All drugs were used in doses of 100 mg/kg. The following drugs were given intramuscularly only: benzylpenicillin (penicillin G), p-aminobenzylpenicillin, methicillin, streptomycin, paromomycin and neomycin. The following drugs were given intramuscularly and orally: D(−)-6-(α-amino-α-phenylacetamido)penicillanic acid plus L(+)-6-(α-amino-α-phenylacetamido)penicillanic acid, and chloramphenicol.

RESULTS

The concentrations of the different antibiotics in the bile after single doses of 100 mg/kg are shown in Table 1. The penicillins were all present in high concentration within 30 min of an intramuscular injection. Excretion proceeded at a high level for a further hour and then diminished. The highest rate of biliary excretion was that of p-aminobenzylpenicillin (over 20% of the dose in 2.5 hr), then D(−)-6-(α-amino-α-phenylacetamido)penicillanic acid and its L(+) isomer, benzylpenicillin and methicillin, in that order. The peak concentrations in the bile were 15 to 90 times higher than the corresponding blood levels.

The other antibiotics tested (Table 1) were present only in minute amounts in the bile. Streptomycin gave the highest yield in this group, but the total excretion

TABLE 1

CONCENTRATIONS (μG/ML.) (AND STANDARD ERRORS) OF ANTIBIOTICS IN THE BILE OF RATS DOSED WITH 100 MG/KG INTRAMUSCULARLY

(−)-6-(α-Amino-α-phenylacetamido)penicillanic acid=BRL 1341, the L(+) isomer=BRL 1060

Hr after injection	Penicillins								Chloramphenicol
	Benzyl	*p*-Aminobenzyl	BRL 1060	BRL 1341	Methicillin	Streptomycin	Neomycin	Paromomycin	
0·5	695±148	2,729±115	340±77	1,385±161	377±57	6·0	3·0	4·0	1·2
1·5	297±146	2,035±129	446±61	1,344±224	399±60	10	2·7	5·0	7·0
2·5	24	414±179	312±58	984±282	145±105	4·0	1·7	3·0	1·0
3·5			88		35			4·0	
4·5			49		14			Trace	
5·5			26						
5·5–20	43	43	23	30		0	0·6	0	0
Plasma (1 hr)	50	40	35	16	29	66		80	20

(0.08% of the dose) and the peak concentrations (10 μg/ml. or less) were very low indeed compared with any of the penicillins (Fig. 1). Peak values, occurring at 0.5 to 1.5 hr after injection, were much lower than the corresponding plasma levels. Streptomycin and paromomycin were not detectable after this period, but a trace of neomycin was present in the samples of bile collected overnight. When given intramuscularly, chloramphenicol was not detectable after 2.5 hr, but, when given orally, this drug was excreted more slowly in the bile, producing a higher concentration (2.5 μg/ml.) in the overnight sample than in earlier samples.

The concentrations produced in the bile by the various penicillins were, without exception, vastly higher than the concentrations required for the inhibition of sensitive organisms, whereas the concentrations produced by the other antibiotics were, in this respect, marginal and transient. p-Aminobenzylpenicillin gave the highest concentrations; and this compound, together with D(−)-6-(α-amino-α-phenylacetamido)penicillanic acid, yielded concentrations which were significantly higher than the three other penicillins.

Having been once excreted in the bile, the penicillins were reabsorbed and re-excreted into the duodenum. In the case of p-aminobenzylpenicillin, D(−)-6-(α-amino-α-phenylacetamido)penicillanic acid and its L(+) isomer, re-excretion produced in the bile concentrations which, though much lower than those detected in the primary excretion, were still sufficiently high (10 μg/ml. or more) to be bactericidal toward a wide range of pathogens, including the Salmonellae. In the case of the two acids, bactericidal levels were detected in the bile after oral as well as intramuscular administration, though the peak values (400 μg/ml.) were lower, and were not attained until about 3 hr after dosing. Reabsorption and re-excretion continued so that bactericidal levels (6 to 30 μg/ml.) were still detectable in the bile between 3 and 15 hr. Benzylpenicillin, p-aminobenzylpenicillin and methicillin, being acid-labile, were not tested in this way.

DISCUSSION

It is clear from the results that the various penicillins are rapidly and directly transferred from the blood stream into the bile in high concentration in the rat,

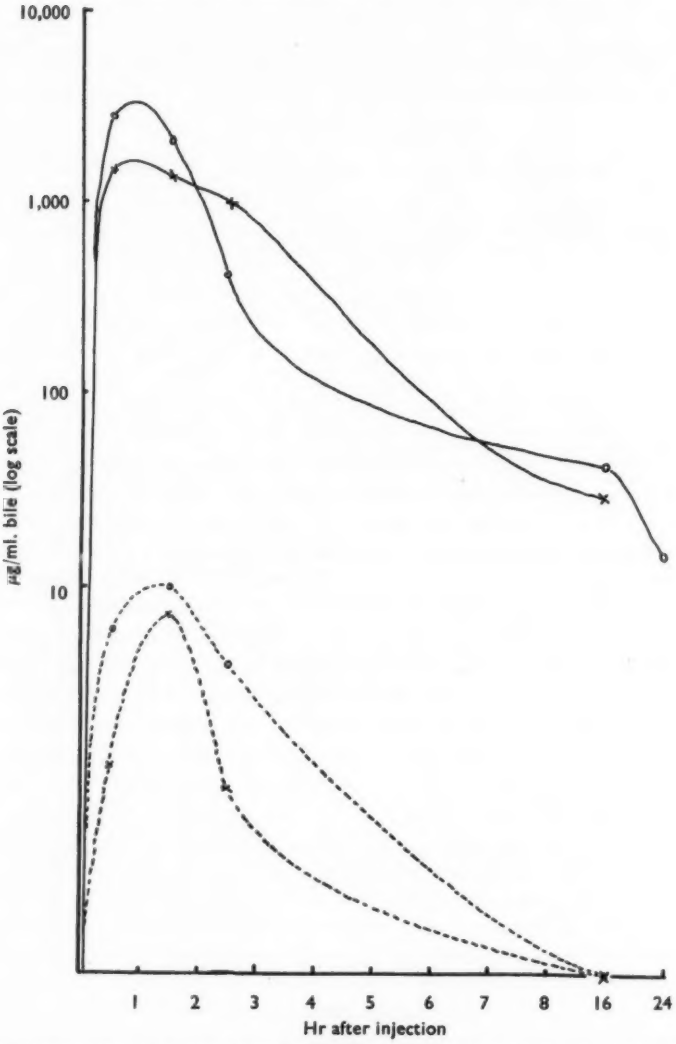

Fig. 1. Biliary excretion (µg/ml.) of 2 penicillins, streptomycin and chloramphenicol in rats given 100 mg/kg intramuscularly. O——O *p*-Aminobenzylpenicillin. X——X D(−)-6-(α-Amino-α-phenylacetamido)penicillanic acid. O - - - O Streptomycin. X - - - X Chloramphenicol.

and that reabsorption and re-excretion of this moiety maintain a bactericidal level of drug in the plasma, bile and upper part of the gut for some hours after a single dose. In contrast, streptomycin, paromomycin, neomycin and chloramphenicol reach lower levels in the bile than in the plasma ; these levels, at peak, are only on the threshold of bacteriostatic activity even when the drugs are given intramuscularly ;

when given orally, as in conventional therapy of an alimentary infection, chloramphenicol, which is well absorbed, may just attain a bacteriostatic concentration in the bile; streptomycin, paromomycin and neomycin, being poorly absorbed, can be discounted in this respect.

It is well known that carriers of certain Salmonellae, especially *S. typhi* and *S. paratyphi B*, may harbour the organisms largely or entirely in the biliary tract, and it is tempting to speculate that the traditional difficulties of eradicating such infection might be due to lack of concentration of eligible antibiotics in the bile, the failure of chloramphenicol in this respect being especially noteworthy. If this explanation is valid, two of the penicillins described here, D(−)-6-(α-amino-α-phenylacetamido)penicillanic acid and its L(+) isomer, might be more promising in biliary carriers, since, as well as being excreted and re-excreted in vastly higher concentration in the bile, both are more actively bactericidal to Salmonellae than any other eligible antibacterial drugs. If infection is localized in the lower bowel as well as in the biliary tract, this reasoning will not apply, since reabsorption will prevent an inhibitory quantity of drug reaching the ileum; in this event, it would seem necessary to administer the drug simultaneously in enteric-coated capsules to ensure its liberation at lower levels in the gut.

The results obtained here in rats may or may not be applicable to man; but it is known that penicillin G is excreted in human bile (Goodman & Gillman, 1955; Sollmann, 1957) and that methicillin behaves similarly (Stewart, Harrison & Holt, 1960). It seems reasonable, therefore, to expect that D(−)-6-(α-amino-α-phenylacetamido)penicillanic acid and its L(+) isomer, which are similar pharmacologically, will behave in the same way in man. The rat has no gall-bladder and the drug levels recorded in the bile derive therefore from direct excretion. It is possible that, in man, a higher local level will result from the considerable concentration which normally affects all other components of the bile in the gall-bladder.

The high rate of immediate excretion of these various penicillins in the bile, coupled with their lack of organic toxicity, suggests that the synthetic penicillins as well as penicillin G do not invoke detoxifying mechanisms in the liver and are therefore equally well eliminated and equally acceptable to the body.

Grateful acknowledgment is made to Dr J. M. Barnes for facilities in his laboratory; Mrs Jean A. White for skilled technical assistance; Beecham Research Laboratories for supplies of D(−)-6-(α-amino-α-phenylacetamido)penicillanic acid and its L(+) isomer and methicillin; and to the Connaught Research Laboratories (University of Toronto) for a supply of p-aminobenzylpenicillin.

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A COMPARISON OF TESTS FOR ANTIFIBRILLATORY ACTION

BY

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The object of the experiments was twofold: first, to choose the most satisfactory test for antifibrillatory action; secondly, to place several drugs with reputed antifibrillatory activity in an order of potency as a preliminary to investigating their mode of action. Measurements were made on isolated rabbit atria at 34° C of (1) the maximum driving frequency the atria would follow, (2) conduction velocity, (3) contractions, and of the threshold for the production of (4) extrasystoles, (5) flutter and (6) fibrillation. Log dose-response curves were plotted for quinidine, papaverine, procaine, dibenamine and procaine amide. The maximum frequency test and fibrillation threshold test gave similar results with all the drugs, and the results gave the order quinidine 1.0, procaine 0.53, Dibenamine 0.47, papaverine 0.43 and procainamide 0.26. Thresholds for extrasystoles and flutter were much more variable. The regressions relating changes in conduction velocity and contraction to log dose were different from those for maximum driving frequency and fibrillation threshold for procaine, papaverine and dibenamine, but the regressions for quinidine and procaine amide were nearly parallel in all tests. Serpajmaline contained a substance with antifibrillatory activity as great as that of quinidine and with no greater depressant action on contractions.

There is still no satisfactory explanation for the phenomenon of fibrillation, which makes the interpretation of the mode of action of antifibrillatory drugs difficult. Although it has been established that Lewis's hypothesis of a circular sequence of excitation can no longer be maintained in its original form (Scherf & Schott, 1953; Prinzmetal, Corday, Brill, Oblath & Kruger, 1952), and that flutter and fibrillation can originate from an ectopic focus, it remains to be discovered why a particular region of heart muscle should suddenly become an ectopic pacemaker, and why uncoordinated activity should spread from such a focus over the whole of the atria or ventricles. It is a fact, however, that fibrillation can be precipitated by a variety of methods, including the focal injection of aconitine (Scherf, 1947), the combined action of cholinergic drugs and electrical stimulation (Burn, Vaughan Williams & Walker, 1955), or by suitably timed electrical stimulation alone (Dipalma & Schults, 1950; Brooks, Hoffmann, Suckling & Orias, 1955; Szekeres & Lénárd, 1960), and that fibrillation, both clinical and experimental, can be stopped or prevented by a number of substances of quite different chemical structure.

Compounds with allegedly antifibrillatory action have usually been compared (Dawes, 1946) with quinidine, as the parent antifibrillatory drug. Quinidine was at first claimed to prolong the absolute refractory period of cardiac muscle (Lewis, Drury, Iliescu & Wedd, 1921) and then shown not to do so (Love, 1926). Yet there

is no doubt that quinidine reduces the frequency at which cardiac muscle can be induced to follow strong electrical stimuli, that is, it increases the "effective" refractory period (Lewis & Drury, 1926), and also increases the threshold to stimulation by electrical current or an ectopic focus (Scherf & Schott, 1953; Prinzmetal, Corday, Brill, Oblath & Kruger, 1952).

Authors still do not agree how antifibrillatory drugs work, however (Vaughan Williams, 1958b; West & Amory, 1960; Burn, 1960). Any hypothesis concerning their mode of action would obviously be strengthened if it could be shown that all drugs with known antifibrillatory effects had certain properties in common. It would first be necessary, however, to define what constituted an antifibrillatory drug. Many different tests for antifibrillatory action have been described, but there has not been, so far as we know, any previous attempt to compare them quantitatively. The object of the experiments described here was to place several substances, with reputed antifibrillatory activity but of different chemical structure, in an order of potency by comparing their relative performance in several different tests. Any other action they might have in common, for example, a depression of the rate of rise of the action potential in the absence of any fall in resting potential or prolongation of repolarization, as has been described by Szekeres & Vaughan Williams (1961), would provide some evidence for the relevance of this effect to their antifibrillatory action.

METHODS

Isolated rabbit atria were suspended horizontally in a water-jacketed bath, the temperature of which was kept at 34° C, and were supplied with modified Locke solution as previously described (Vaughan Williams, 1958a; Szekeres & Vaughan Williams, 1961). Re-oxygenation of the bath fluid was carried out in a subsidiary rapid circulation, fluid being continuously withdrawn, oxygenated and returned to the bath. Contractions were recorded by an RCA 5734 transducer, and displayed on one beam of a Dumont 322 oscilloscope. Action potentials for the measurement of conduction velocity were recorded by two pairs of small bipolar electrodes on the left and right atria, and were displayed on the other beam. The traces were photographed on film and measured subsequently with a projector. The stimulator was constructed by one of us and consisted of two channels, each comprising frequency, delay, duration and output stages, which could be locked together in various ways or could be operated independently. In the present experiments the output stages were employed as constant current devices over the range 20 μA to 25 mA, so that small changes of resistance in the tissue did not affect current strength. Stimulator A was used to provide the fundamental driving frequency and to trigger the oscilloscope sweep, and was connected to a pair of small platinum electrodes via an isolation transformer. Stimulator B was used to produce extra-systoles, flutter and fibrillation, and was directly coupled to a pair of silver-silver chloride disc electrodes of 2.8 mm diameter, placed above and below the left atrial tip. Drug concentrations have been expressed as w/v salt. Quinidine sulphate, papaverine sulphate and procaine hydrochloride were obtained from British Drug Houses, Dibenamine from Smith Kline and French, and procainamide from Squibb. The serpajmaline and ajmaline were provided by the kindness of Professor S. Siddiqui, of the Pakistan Council of Scientific and Industrial Research.

RESULTS

Experimental procedure. The actions of papaverine, quinidine sulphate, procaine hydrochloride, procainamide and Dibenamine were examined on a total of 41 pairs of rabbit atria and ajmaline and serpajmaline on three. The following measurements were made:

(1) The threshold for electrical excitation. Stimulator A was adjusted to a duration of 1.0 msec and a frequency of 180/min, which was in nearly every case faster than the spontaneous frequency. On a few occasions when the spontaneous frequency was faster than this, the stimulation frequency was set a little higher than the spontaneous frequency. Increasing current was then passed until the atria began to follow the stimulator. The thresholds were in the range 50 μA to 420 μA.

(2) Maximum driving frequency. The stimulus strength was raised to 2 mA and the frequency of stimulator A increased until the atria would no longer follow every stimulus.

(3) Threshold for extrasystoles. The preparation was again driven by A at 180/min. Stimulator B was set to a duration of 3 msec and a frequency of 600/min. The strength was increased until an extrasystole was observed.

(4) Threshold for flutter. The stimulus strength of B was further increased until a condition of flutter was induced, that is, until the atria broke away from both stimulators and beat at a fast regular frequency with co-ordinated contractions. The threshold for the induction of this effect proved to be sharp and reproducible.

(5) Threshold for fibrillation. The strength of the stimuli from stimulator B was further increased until flutter changed into fibrillation, that is, until the contractions became incoordinated. Here again a sharp and reproducible threshold was obtained. The length of time for which the fibrillation continued after cutting off stimulator B was very variable and could not be used as a test of any value.

(6) Conduction velocity. Action potentials from left and right atria were photographed while the atria were being driven at frequencies of 142 (if the spontaneous frequency was less than this), 180, 196, 244, 302, 367, 464/min, in so far as they would still follow. The change in conduction velocity after administration of the drug was then calculated separately at each frequency as a percentage of the control conduction velocity at that frequency. It was found that the percentage reduction in conduction velocity was always relatively a little greater at the higher frequencies, that is, the sensitivity of conduction velocity as a test of drug action was increased at the higher frequencies. For the purpose of plotting the log dose-response curve in Fig. 3, the percentage change was calculated as the mean change at all the frequencies measured.

(7) Contractions.

Experimental observations. Refractory period is affected by a number of factors, including the fundamental driving frequency and the strength of the conditioning stimulus (Dawes & Vane, 1956). Measuring the maximum frequency at which atria will follow a stimulus is not truly, therefore, a test of the effective refractory period, but Dawes (1946) showed that it was of great practical value in testing synthetic substitutes for quinidine. The results of this test are presented in Fig. 1, and show that quinidine is the most and procaine amide the least active substance. The parallelism of the dose-response curves is reasonable.

The measurements of the threshold for extrasystoles and for flutter put the drugs in the same order, but the dose-response curves were neither so straight nor so parallel as in the maximum frequency test. Consequently it has not been considered

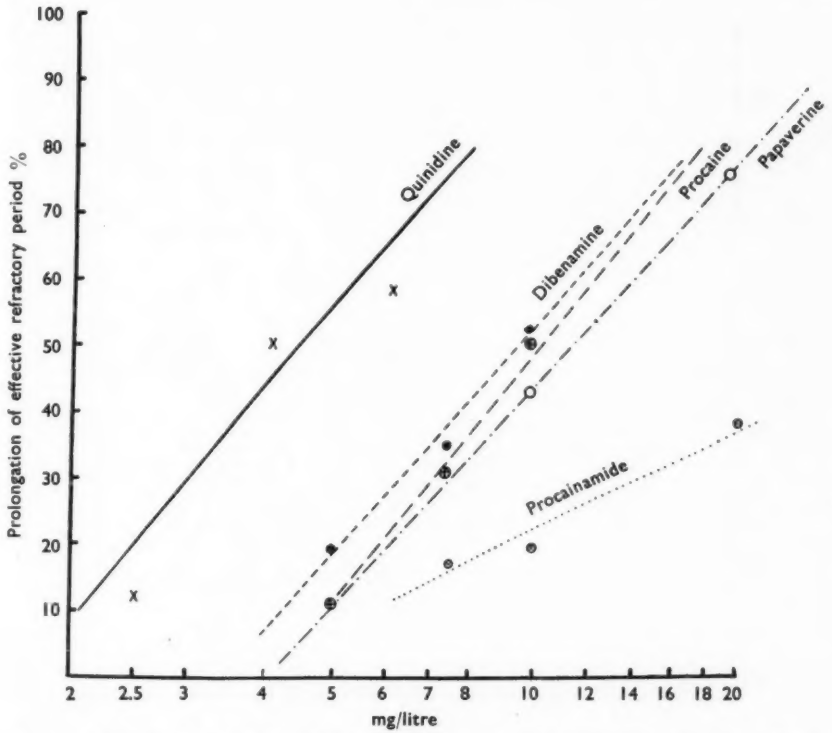

Fig. 1. Prolongation of effective refractory period. In this and subsequent figures the ordinates show % change, the abscissae drug concentration in mg/l. on a log scale. Crosses: quinidine. Dots: Dibenamine. Open circles: papaverine. Crossed circles: procaine. Dotted circles: procainamide.

worth plotting the results, as these two tests were obviously inferior. Measurements of the fibrillation threshold, on the other hand, gave results very similar to measurements of increase in effective refractory period in the maximum frequency test, and have been plotted in Fig. 2.

Conduction velocity was reduced by all the drugs, and, since the measurement of the distance between photographed action potentials is easy, accurate and entirely objective, it was hoped that conduction velocity measurements might afford a useful antifibrillatory test. The decreases in conduction velocity were relatively greater at the higher driving frequencies, which improved the discrimination. The results have been plotted in Fig. 3. It is at once apparent that the dose-response curves for Dibenamine, procaine and papaverine have quite different slopes from those for quinidine and procainamide. The implication is that, although all drugs with antifibrillatory action depress conduction velocity, the factors controlling conduction velocity are complex and are not necessarily affected to the same extent as the factors governing susceptibility to fibrillation. For this reason measurements of

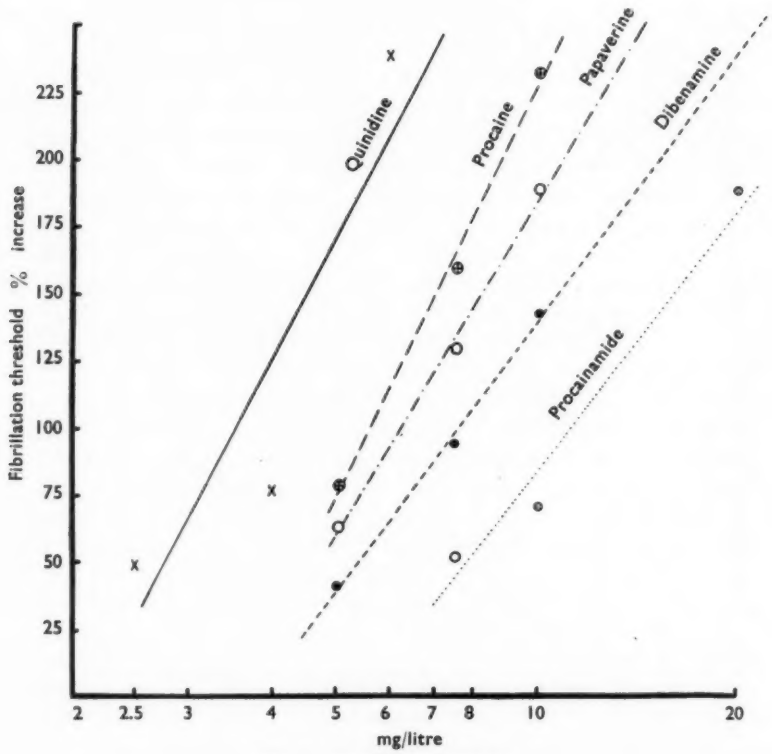

Fig. 2. Fibrillation threshold.

conduction velocity cannot be regarded as a satisfactory test for antifibrillatory activity. Finally, measurements of contraction also indicated a difference in slope in the dose-response curves for Dibenamine and procaine as compared with quinidine and procainamide (Fig. 4). Papaverine increased the contractions.

The least departures from parallelism in all the tests were between the results obtained with quinidine and procainamide, and it is therefore probable that these two drugs act in the same way. On fibrillation threshold procainamide had 0.32 the activity of quinidine and on contractions 0.29 the activity, but the difference between these two ratios was not statistically significant. These tests, therefore, show no therapeutic advantage of procainamide over quinidine. In Table 1 the mean doses of each drug required to produce a 100% increase in fibrillation threshold have been given in the first column. The second column indicates the mean changes in the size of contractions produced by these drug concentrations, and the third and fourth show changes in effective refractory period and conduction velocity respectively.

The fact that papaverine increased contractions although it had an antifibrillatory action, and that Dibenamine caused a relatively greater depression of contractions

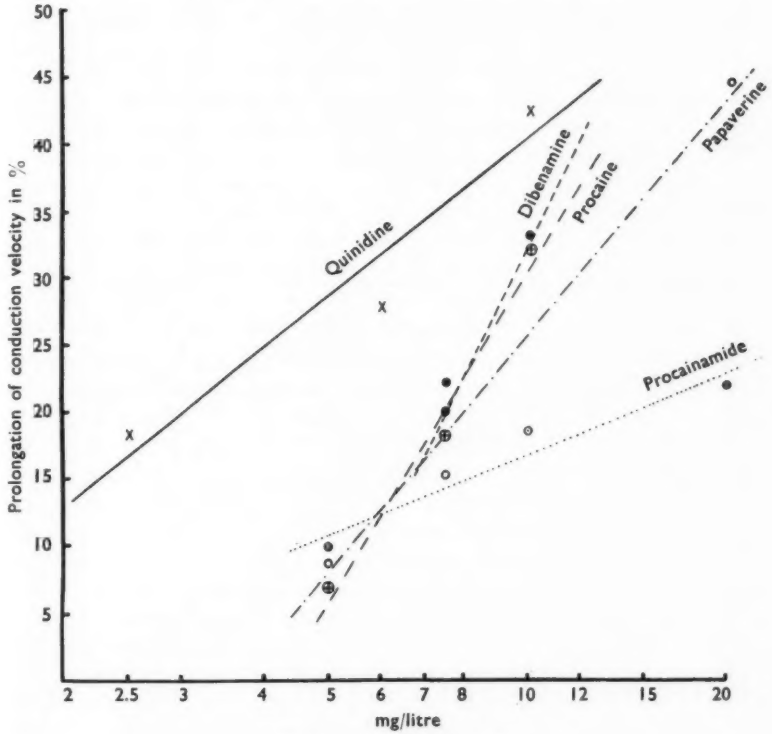

Fig. 3. Conduction velocity.

than the other drugs in relation to its antifibrillatory activity, indicated that the effects on susceptibility to fibrillation and contraction were at least separable, and gave some grounds for believing in the possibility of finding a more specific antifibrillatory compound. Serpajmaline has already been shown to have an antifibrillatory action (Child, Davis, Sharpe, Tomich & Deininger, 1959; Deininger, 1959). Insufficient experiments have been done so far to establish whether the mode of action is similar to that of the other drugs, but we have confirmed that serpajmaline contains a substance with an antifibrillatory action at least as powerful as that of quinidine, and with no more, and probably rather less, depressant action on contractions.

DISCUSSION

The object of the present work was twofold: first, to choose the most satisfactory test for antifibrillatory activity out of several that have been described; secondly, to put several drugs of different chemical structure in an order of antifibrillatory activity as a preliminary to an investigation of their mode of action. A quantitative comparison of quinidine, procaine, papaverine, Dibenamine and procainamide was undertaken, and a few experiments were also done with ajmaline and serpajmaline. Measurements of the maximum frequency at which atria would

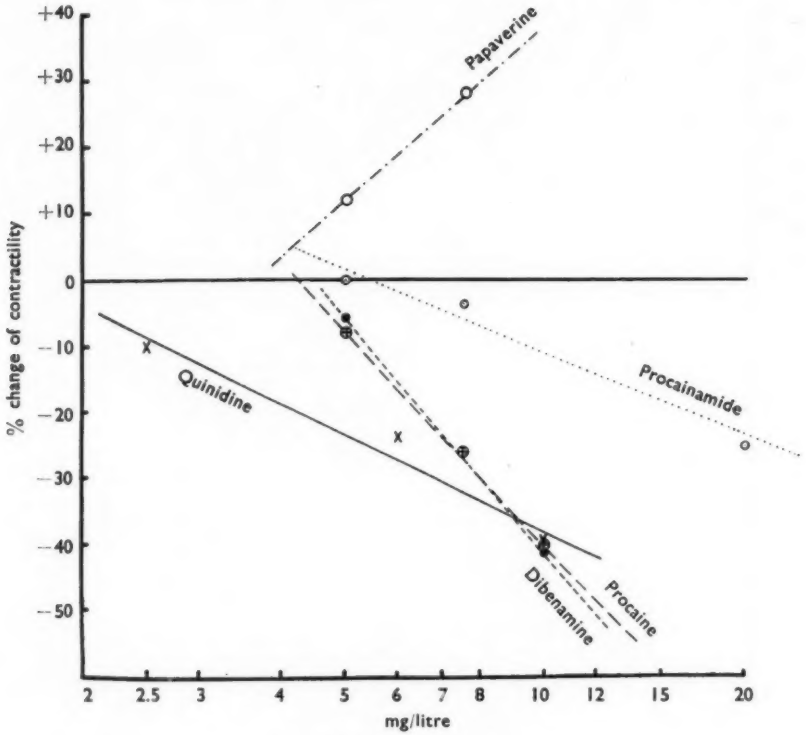

Fig. 4. Contractions.

follow a driving stimulus gave very similar results to measurements of the fibrillation threshold. Linearity of log dose-response and parallelism was much less good in measurements of the threshold for extrasystoles and for the development of flutter, and these tests were regarded as unsuitable for estimating antifibrillatory activity.

For quinidine and procainamide the regressions relating response and log dose were similar in all the tests, and it has been concluded that these drugs act in the

TABLE 1

In column 1 is given the mean concentration required to produce an increase of 100% in the fibrillation threshold. Column 2 shows the change in the magnitude of contractions produced by the concentrations in column 1, and columns 3 and 4 indicate the changes in refractory period and conduction velocity produced by these concentrations

	1 Conc. in mg/l.	2 Change in contraction %	3 Prolongation of refractory period %	4 Change in conduction velocity %
Quinidine	3·52	−14·8	+38	−22·5
Procaine	5·5	−11·5	+18	−11
Papaverine	6·2	+20·7	+21	−13
Dibenamine	7·51	−25·0	+38	−20·5
Procainamide	11·1	−12·5	+24·5	−18

same way, and that by the tests used neither had any advantage over the other. Procaine, papaverine and Dibenamine had very different regressions from quinidine and procainamide in measurements of conduction velocity and contraction amplitude. Dibenamine had a relatively greater depressant action on contractions than the other drugs, while papaverine increased contractions in association with an antifibrillatory action. This separation of effects on contraction from the reduction in susceptibility to fibrillation at least offers some hope of the possibility of obtaining a drug with a higher specificity of antifibrillatory action. Insufficient experiments have so far been done with ajmaline and serpajmaline, so that it is not known whether these substances act in the same way as the other drugs. But it was clear that serpajmaline contained a substance with an antifibrillatory action at least as powerful as that of quinidine, without any greater depressant action on contractions.

Measurements of fibrillation threshold and the maximum frequency at which atria would follow a stimulus gave very similar quantitative results, and, since the latter is an extremely easy test to perform, it is felt that the present work vindicates its use as a screening standard for antifibrillatory action (Dawes, 1946).

A possible explanation for the prolongation of the effective refractory period could be a prolongation of the action potential, but the available evidence is that clinically relevant concentrations of quinidine have no effect or only a trivial effect on the duration of the action potential (Wedd, Blair & Gosselin, 1942 ; Vaughan Williams, 1958b). Further, a prolongation of the action potential would offer no explanation for the other predominant effect of quinidine, a rise in threshold to electrical stimulation. More significance was, therefore, attached to the finding that quinidine, even in concentrations lower than those achieved in the blood of treated patients, caused a great slowing of the rate of the rise of the action potential in the absence of any fall in the resting potential (Vaughan Williams, 1958b). It was suggested that both the prolonged effective refractory period and the raised threshold could be accounted for by an interference by quinidine with the mechanism by which depolarizing ions were carried across the membrane. It has now been confirmed (Szekeres & Vaughan Williams, 1961) that all the drugs studied here also have in common with quinidine the property of greatly reducing the rate of rise of the action potential without affecting the resting potential or prolonging the time taken to repolarize to 50% of full repolarization. High concentrations prolonged the time for full repolarization by a few milliseconds, but this was quite inadequate to account for the large prolongation of the effective refractory period.

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SIMILARITY BETWEEN RECEPTORS RESPONSIBLE FOR THE PRODUCTION OF ANALGESIA AND LENTICULAR OPACITY

BY

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Evidence is presented that the receptors responsible for the mediation of analgesia by morphine-like drugs are similar to those which are involved in the production of a reversible lenticular opacity. The activity of a number of compounds in mice on the lens was closely correlated with analgesic potency in this species. Stereospecificity for isomers with D configuration was demonstrated for both effects. Nalorphine only antagonized the lenticular opacity activity of those drugs the analgesic action of which it abolished.

The appearance of reversible lenticular opacity in rodents by the acute administration of analgesic drugs has been reported by Weinstock, Stewart & Butterworth (1958) and by Weinstock & Stewart (1961). It is generally accepted that these drugs combine with specific receptors in the central nervous system in order to elicit an analgesic response. The evidence for the existence of such receptors is based on the structural requirements for potency (Braendon, Eddy & Halbach, 1955), the importance of stereochemical configuration (Beckett & Casy, 1954; Beckett, Casy & Harper, 1956), and the specific antagonism by nalorphine. As preliminary experiments had indicated that the formation of an opacity was confined to analgesic drugs of the morphine type, a study was undertaken to determine whether the receptors involved in these two actions of morphine-like drugs were similar.

METHODS

Male and female mice of a Pirbright (Albino) strain, weighing 18 to 22 g, were used.

Analgesic activity

The modification of the hot-plate method of Eddy & Leimbach (1953), described by Janssen & Jageneau (1957), was used for measuring analgesic activity.

The reaction times of mice were determined twice before, and 15, 30, 45, 60, 90, 120, and 180 min after subcutaneous injection of the drug. All animals whose mean initial reaction-time exceeded 10 sec, or in which the two readings differed by more than 100%, were discarded. An analysis of the average of the two reaction times (measured to 0.2 sec) of some 2,300 mice, estimated before injection, showed that there was no significant difference between the readings of male and female mice, the mean reaction time of males being 6.0 ± 0.2 sec and that of females 6.0 ± 0.2 sec.

A response was considered positive if the reading after injection was greater than 30 sec, or if it exceeded the initial mean reading by a factor of three or more. The number of such positive responses was noted at each of the various times. The largest number of positive

responses at each dose of each analgesic drug was converted to a percentage and used in the plotting of a dose-response curve from which the ED50 was calculated.

Opacity-producing activity

Mice were placed in containers in groups of 10 and examined at 15, 30, 45, 60, 75, 90, 120, 150, and 180 min after subcutaneous injection of the drug. The number of mice showing any degree of opacity at each of the above times was determined.

All drugs were administered as aqueous solutions of their salts, in a constant volume of 1.0 ml./100 g. The compounds listed in Table 2 were injected subcutaneously in doses ranging from 1 to 20 mg for the opacity tests, and from 0.1 to 10 mg for the measurement of analgesic activity, in increments of 0.2 or of 2 mg until an effect was obtained. If no opacity was produced in doses below 20 mg, and this quantity was not lethal, larger doses were given until 50% mortality occurred.

The ED50 values for opacity and analgesia for all active drugs were determined at the time of maximum activity. At least three doses of each compound were administered. A minimum of 30 mice was used for each dose. The ED50 values and their fiducial limits of error were computed by the method of probit analysis.

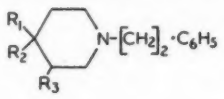
All doses are expressed in mg/100 g body weight.

Structure–activity relationships

A new series of compounds, well suited to the study of structure–activity relationships, was made available for our use by Dr A. H. Beckett, the structures of which are shown in Table 1.

TABLE 1

STRUCTURES OF SOME DERIVATIVES OF 1-PHENETHYLPIPERIDINE



Series and number	R_1	R_2	R_3
A1	C_6H_5–	–OH	–H
A2	*o*–$C_6H_5.C_6H_4$–	–OH	–H
A3	*o*–$CH_3.C_6H_4$–	–OH	–CH_3
A4	*p*–$CH_3.C_6H_4$–	–OH	–CH_3
A5	CH:C–	–OH	–H
A6	N:C–	–OH	–H
E1	C_6H_5–	–O.CO.CH_3	–H
E2	*o*–$CH_3.C_6H_4$–	–O.CO.CH_3	–CH_3
E3	*o*–$CH_3O.C_6H_4$–	–O.CO.CH_3	–CH_3
E4	*m*–$CH_3.C_6H_4$–	–O.CO.CH_3	–CH_3
E5	*p*–$CH_3.C_6H_4$–	–O.CO.CH_3	–CH_3
E6	*o*–$CH_3.C_6H_4$–	–O.CO.C_2H_5	–CH_3
E7	*m*–$CH_3.C_6H_4$–	–O.CO.C_2H_5	–CH_3

RESULTS

The ED50 values for analgesic and opacity-producing activity of a number of established drugs, as well as those of the new A and E series, are shown in Table 2, with the fiducial limits of error and slopes of regression lines expressed in mg/100 g of the free base. The LD50 of most is given for comparison.

The results show that all compounds which produced an opacity had analgesic activity. However, apomorphine and A4 had no effect on the lens although they had apparent analgesic activity. As the ED50 for opacity was usually about five

TABLE 2

A COMPARISON OF THE ED50 VALUES FOR ANALGESIA AND OPACITY OF A NUMBER OF COMPOUNDS

All doses are in mg/100 g free base. The numerals in parentheses indicate the % effect obtained by the given dose when it was not possible to obtain an ED50. With some substances no analgesia and no lens opacity activity was found even with the quantities designated (−)25 and (−)50 as with nalorphine

Compound	ED50 analgesia	Fiducial limits %	Slope "b"	ED50 opacity	Fiducial limits %	Slope "b"	LD50
Morphine	0·43	80·4–121	1·58	3·06	87·5–114	1·72	39·6
Codeine	3·73	70·8–131	1·52	14 (30%)	—	—	14·2
Dihydrocodeine	2·84	79·6–121·0	1·87	12 (40%)	—	—	19·4
Diamorphine	0·174	75–126	1·58	0·37	82–118·8	3·13	22·8
Normorphine	5·79	67–121·7	4·69	11 (30%)	—	—	10·4
Nalorphine	(−)25	—	—	(−)50	—	—	51·2
Levorphanol	0·170	80·3–121·2	1·84	0·34	82·6–117·8	3·4	10·7
Levomethorphan	0·58	77·5–129·6	1·87	2·50	79·4–121·0	2·94	7·83
Levallorphan	(−)20	—	—	(−)25	—	—	—
Apomorphine	3·01	75·8–132·2	3·46	(−)10	—	—	14·9
Papaverine	7·71	92–108·4	10·87	15·3	87–114·6	5·76	20·3
Pethidine	1·84	77·5–129·6	2·07	3·77	81·6–118·4	3·22	20·8
(±)-Methadone	0·40	86·8–114·9	2·61	0·80	89·5–111·7	3·54	3·56
Dextromoramide	0·078	75·2–126·8	2·1	0·189	78–129	3·01	14·0
E1	0·213	79·6–121·2	1·8	0·910	83·8–114	2·28	—
E2	0·080	89·6–111·7	1·95	0·443	90–109·2	3·24	20·9
E3	0·272	69·2–132·8	2·0	0·564	69·6–132	3·01	—
E4	0·577	74·6–128	1·7	2·78	87·2–114·2	3·99	—
E5	0·702	77·5–129	1·91	3·34	80·3–121	3·07	—
E6	0·400	80·2–121	1·27	1·184	85·5–117·2	2·82	—
E7	2·65	75·2–126·8	2·1	6·29	80·6–121·2	3·10	—
A1	(−)10	—		(−)10	—	—	15·0
A2	(−)5·0	—		(−)7·5	—	—	8·6
A3	1·45	89–113	4·43	8·2	88·3–113·5	6·98	9·46
A4	1·175	64–137	4·9	(−)5·0	—	—	6·0
A5	(−)15·0	—		(−)20·0	—	—	37·5
A6	2·5	68·5–131·8		3·0 (25%)	—	—	6·3

times that for analgesia, it was possible that an opacity was not seen with these two compounds because the dose necessary was above the LD50.

With the derivatives of morphine, including the morphinan series, activities in two tests for each effect were similarly related. Changes in potency which accompanied alterations in chemical structure in most of the 1-phenethylpiperidine derivatives (A and E series) were reflected similarly in the two activities. As in the morphine series, and in many other synthetic analgesic drugs, esterification of the alcoholic hydroxyl group markedly increased activity. The position of the methyl substituent in the 4-phenyl ring appears to be of importance, producing the most active member when it is in the *ortho* position (E2). The acetoxy derivatives were more potent than the corresponding propionyloxy derivatives.

The relative activities of the various groups of compounds in the two tests are shown in Table 3, expressed as a ratio of the two ED50 values. The drugs are also listed in order of their efficiency in producing opacity and analgesia.

With the exception of that of morphine, the ratios of activity in the two tests fall into two distinct groups, with means of 2.13 ± 0.13 and 4.84 ± 0.45, respectively.

The relatively high ratio of the two ED50 values of morphine appears to be due to the fact that the slope of the regression line for its activity on the eye is

TABLE 3
RATIOS OF ACTIVITY OF COMPOUNDS ON THE EYE TO THAT OF ANALGESIC ACTIVITY

Compound	ED50 opacity/ ED50 analgesia	Order of activity	
		Analgesia	Opacity
Levorphanol	2·0	3	2
Papaverine	2·0	20	>17
(±)-Methadone	2·0	7	6
Pethidine	2·05	14	13
E3	2·08	6	5
Diamorphine	2·12	4	3
E7	2·38	16	15
Dextromoramide	2·40	1	1
A6	>1·5	15	14
Normorphine	>1·9	19	>17
Levomethorphan	4·3	11	9
E1	4·27	5	7
E6	4·62	8	8
E5	4·75	12	12
E4	4·8	10	10
E2	5·5	2	4
A3	5·65	13	16
Codeine	>3·75	18	>17
Dihydrocodeine	>4·2	17	>17
Morphine	7·1	9	11

much less than those of other drugs. The dose-response line for analgesic activity, however, does not so differ in slope. The typical regression lines obtained in the opacity test and those for analgesia are shown for a number of compounds in Figs. 1 and 2.

It had been noted that the time of onset of the opacity and that of maximum activity occur much later with morphine than for all other drugs. With most compounds the peak activity is reached 30 to 40 min after subcutaneous injection but is not seen until 75 to 90 min with morphine, particularly when larger doses are given. This may explain the shallow regression line seen with this drug.

At present there is no obvious explanation of why the remaining drugs should fall into either one of the two distinct groups. The route of administration does not appear to make any difference to the ratios of the ED50 values for the two actions. The analgesic activity and opacity-producing potencies of methadone, pethidine and morphine were determined when these drugs were given intraperitoneally and by mouth in addition to the subcutaneous route. The ratios of the ED50 values in the two tests are shown in Table 4.

It appears that the time of peak activity may influence the ratio to some extent, since the ratios for morphine were slightly reduced when maximum activity occurred earlier.

Stereochemical considerations

In all instances in which a compound had been resolved into optical enantiomorphs, on testing one was much more active than the other of each pair, and often the whole of the analgesic activity of the racemic mixture resided in one of the isomers (Beckett *et al.*, 1954). If it could be demonstrated that in a pair of optical

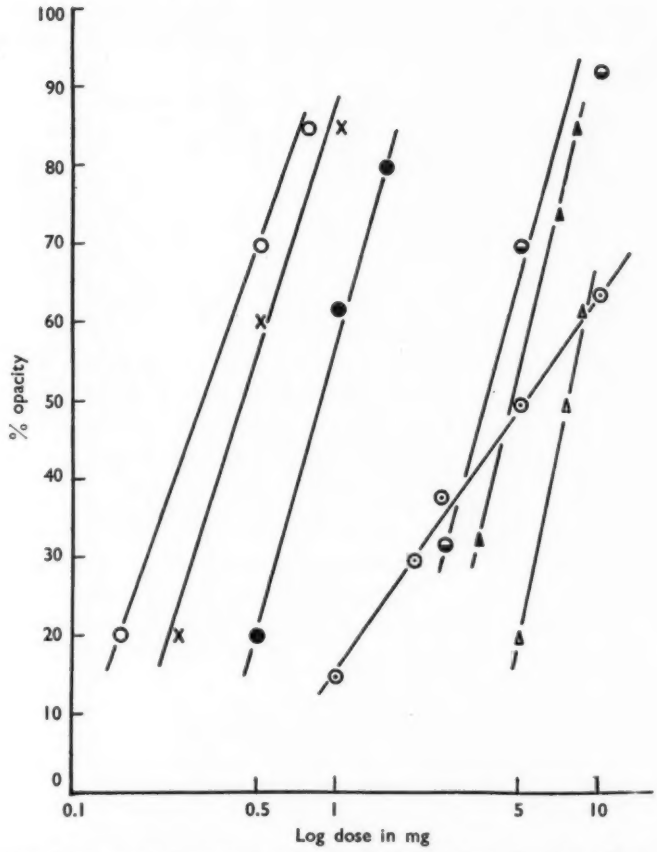


Fig. 1. Relation of opacity-producing activity to dose. Abscissa: log dose in mg/100 g body weight. Ordinate: number of mice showing opacity at a given time expressed as a percentage. ○—○ E2; X—X diamorphine; ●—● methadone; ○—○ morphine; ◒ E7; △—△ A2; ▲—▲ pethidine.

enantiomorphs the isomer with greater analgesic potency was also the more active in producing an opacity, then the receptor for lens opacity is probably similar to that involved in the mediation of analgesia.

Owing to the great difficulty in achieving a pure chemical separation of racemates into their optical constituents, it is almost impossible to obtain isomers in sufficient quantity to carry out adequate pharmacological tests. However, the ED50 values for analgesic and opacity-producing activity were determined for a number of isomeric pairs (Table 5).

The L(+)-isomers, dextrorphan and dextromethorphan, were completely devoid of either activity in the amounts used. With all other pairs, the more potent analgesic

drug was found to be the more effective one in producing an opacity. Furthermore, the ratios for the activity of one isomer to that of the other were constant for the two actions.

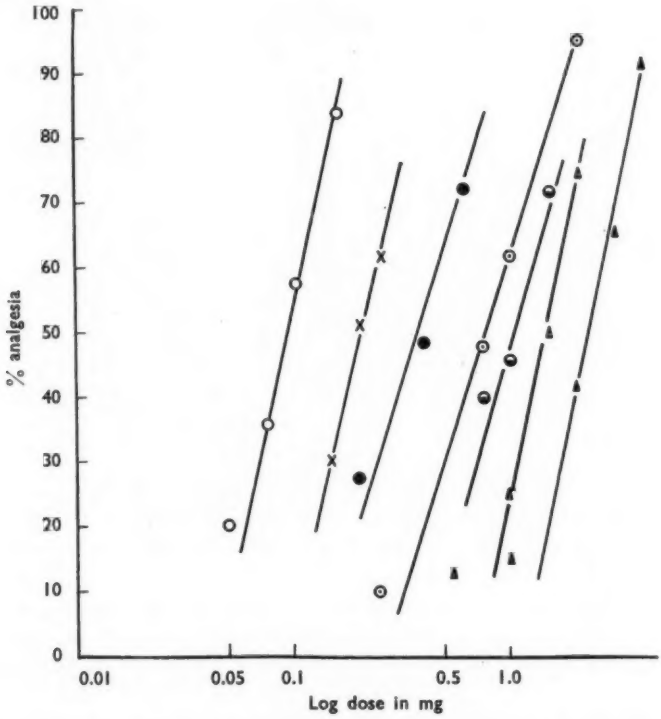


Fig. 2. Relation of analgesic activity to dose. Abscissa: log dose in mg/100 g body weight. Ordinate: Analgesic activity expressed as a percentage. ○—○ E2; X—X diamorphine; ●—● methadone; ⊙—⊙ morphine; ◒—◒ E7; △—△ A2; ▲—▲ pethidine.

TABLE 4

EFFECT OF ROUTE OF ADMINISTRATION ON ACTIVITY RATIOS AND TIME OF ONSET OF OPACITY

Drug	Route	ED50 opacity/ ED50 analgesia	Opacity. Time of max. activity
Morphine	s/c	7·2	75–90 min
	i/p	6·1	60
	o/r	5·8	45–75
Methadone	s/c	2·0	30–40
	i/p	3·2	30–40
	o/r	1·8	20–45
Pethidine	s/c	2·05	30
	i/p	2·3	20–30
	o/r	2·0	30

TABLE 5

COMPARISON OF THE ACTIVITY OF PAIRS OF OPTICAL ISOMERS

Drug	Optical rotation	Configuration	Analgesia (mg/100 g)	Opacity (mg/100 g)	Activity ratios: D:L analgesia	Activity ratios: D:L opacity
(−)-Methadone	−	D	0·09	0·2	1 : 22	1 : 25
(+)-Methadone	+	L	2·0	5·0		
Levomethorphan	−	D	0·76	3·3	—	—
Dextromethorphan	+	L	>10·0	>10·0		
Levorphanol	−	D	0·30	0·6	—	—
Dextrorphan	+	L	>10·0	>10·0		
Dextromoramide	+	D	0·087	0·29	1 : 98	1 : 90
Levomoramide	−	L	8·5	26·0		

Nalorphine antagonism

Nalorphine is generally believed to reduce or prevent the analgesic action of morphine-like drugs by competing successfully for the receptor involved (Lasagna, 1954 ; Seibert & Huggins, 1953 ; Landmesser, Cobb & Converse, 1953).

Doses of the active drug were chosen that gave positive effects in both the opacity and hot-plate test, in approximately 70% of the animals. Even though nalorphine can antagonize many times its own weight of analgesic drug (Orahovats, Winter & Lehman, 1954), similar weights of nalorphine and the analgesic agent were given to ensure that any antagonism would be detected. The active drugs were injected subcutaneously immediately after nalorphine. The results in Table 6 show that nalorphine only prevented the production of an opacity by those compounds whose analgesic action it abolished.

TABLE 6

EFFECT OF NALORPHINE ON OPACITY AND ANALGESIA

(+) Complete antagonism. (−) No reduction of effect. (0) Action absent, therefore not tested

Drug	Antagonism by nalorphine: Analgesia	Antagonism by nalorphine: Opacity
Morphine	+	+
Codeine	+	+
Dimorphine	+	+
Dihydrocodeine	+	+
Normorphine	+	+
Levorphanol	+	+
Levomethorphan	+	+
Pethidine	+	+
E1	+	+
E2	+	+
E3	+	+
E4	+	+
E5	+	+
E6	+	+
E7	+	+
Papaverine	−	−
Apomorphine	−	0
(−)-Methadone	+	+
(+)-Methadone	−	−
Dextromoramide	+	+
Levomoramide	−	−
A3	−	−
A6	−	−
A4	−	0

DISCUSSION

These results show that the production of a reversible lenticular opacity is a characteristic action of analgesic drugs of the morphine type. Of the compounds tested, only those which produced an opacity had analgesic activity. It would be of great interest to know whether this relation will continue to be true when more new analgesic substances of widely differing chemical constitution are synthesized.

The hot-plate test, as a method of assessment of analgesia, does not distinguish specific analgesic drugs, such as morphine and pethidine, from several other central nervous depressants. For example, chlorpromazine was more active than methadone in the hot-plate test, but it is devoid of action on the lens (unpublished observation). Thus, although apomorphine and A4 showed some analgesic potency in the hot-plate test and had no opacity-producing activity, the failure of nalorphine to antagonize the effect of these drugs indicates that such analgesia as was found probably resulted from a depressant action at some other site (or sites) in the central nervous system and not through a combination with the specific analgesic receptor. However, papaverine, A3 and A6 did have some effect on the lens, but the doses required were in the lethal range, and neither the opacity nor the analgesia were antagonized by nalorphine. It is thus also clear that these compounds could hardly be producing either action by combining with the specific analgesic receptor.

It is therefore suggested that this action on the lens can be used as the basis of a new method of screening potential analgesic drugs. This test has several advantages over existing screening procedures. No special apparatus is required and there is no dependence upon the subjective response of a small animal to a supposedly painful stimulus, the response to which can be very variable and often difficult to assess. Unlike methods which involve the measurement of reaction time, this test can distinguish between the morphine-like analgesics and other central depressant drugs. Fewer animals are needed for an accurate assessment of potency, and the slopes of a quantal assay procedure are significantly greater in the opacity test than in the hot-plate method (see Table 2).

The results of the opacity test in mice permit selection of compounds for study by other, more specific tests. If an opacity is produced, antagonism by nalorphine should next be ascertained. This will give further evidence that the compound is active similarly to morphine and its congeners.

Many workers have directed their efforts to finding a compound the analgesic activity of which is at least equal to that of pethidine, but which is non-addictive. So far, all of the known potent analgesic drugs whose actions are qualitatively similar to those of morphine have been found to be addictive. Also, in each case, characteristic antagonism was exhibited by nalorphine. It is therefore very likely that, once nalorphine antagonism of the opacity has been demonstrated, further investigation would fail to yield a non-addictive analgesic drug.

The similarity of the receptors responsible for the formation of the opacity to those involved in the mediation of an analgesic response is strikingly confirmed by the difference in activity found with optical isomers. This is further emphasized by the finding that the ratios of potency of one isomer to its optical enantiomorph

were the same for each action. In addition, these findings show that the active substance which combines with the receptor must be either the unchanged drug or a metabolite whose configuration is identical to that of the compound from which it is derived.

Although many theories concerning the mode of action of analgesic drugs stress the part played by specific receptors, their exact location remains obscure. On the other hand, the effect on the lens provides a readily accessible site for the study of the interaction between morphine-like drugs and their receptors.

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Brit. J. Pharmacol. (1961), **17**, 442–450.

TISSUE AMINE LEVELS AND SYMPATHETIC BLOCKADE AFTER GUANETHIDINE AND BRETYLIUM

BY

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A single dose of guanethidine produces a substantial, long-lasting depletion of tissue catecholamines in the rat, whereas a similar dose of bretylium has no effect. Both drugs produce block of the eserine-induced sympathetic pressor effect. Block by guanethidine is induced more rapidly than is amine depletion. When amine depletion is maximal, a noradrenaline infusion is capable of restoring the response to eserine, but no restoration of the response to eserine occurs after noradrenaline infusion into bretylium-treated rats. Catecholamine levels in isolated tissues are not reduced when complete block of sympathetic nerve stimulation has been produced by guanethidine. It is suggested that guanethidine possesses a primary bretylium-like, and a secondary reserpine-like, blocking action. Guanethidine produces a transient lowering of intestinal 5-hydroxytryptamine, and this coincides with increased intestinal motility.

The mechanism of the anti-hypertensive action of guanethidine and bretylium appears to be dissimilar from that of other agents in clinical use. Both drugs impair postganglionic adrenergic transmission and only possess a slight and transient ganglion blocking activity. Neither antagonizes the actions of adrenaline or noradrenaline. It has been suggested that they prevent the liberation of adrenergic transmitter from the nerve endings (Maxwell, Mull & Plummer, 1959; Maxwell, Plummer, Povalski & Schneider, 1960; Maxwell, Plummer, Schneider, Povalski & Daniel, 1960; Boura & Green, 1959). Guanethidine, in contrast to bretylium, depletes tissue catecholamine levels, and the suggestion has been made that this ability is related to the sympathetic blockade (Cass, Kuntzman & Brodie, 1960; Shepherd & Zimmermann, 1959; Burn, 1961). It was decided, therefore, to test this latter hypothesis by comparing the rate of onset and duration of sympathetic block with the rate and duration of amine depletion. This was done using a single dose of guanethidine in intact animals and some isolated tissues. Comparison was made with bretylium in some of the experiments. 5-Hydroxytryptamine levels in the tissues of animals after treatment with these agents were also measured.

METHODS

Male rats, Wistar strain, 160 to 210 g body weight, were given a single dose, by subcutaneous injection, of 15 mg/kg of guanethidine or bretylium, or of 0.9% sodium chloride solution. Six rats from each group were killed at various time intervals after injection. Heart, spleen

and small intestine were removed for assay of catecholamine or 5-hydroxytryptamine levels. Tissues were rinsed with water, blotted on filter paper, weighed and stored at −10° C until required.

Extraction and assay. Tissues were homogenized in 0.01 N hydrochloric acid and extracted with sodium chloride and acid-saturated butanol, which excludes dopamine. The amines in an aliquot of the butanol were returned to a small volume of 0.01 N hydrochloric acid by shaking with heptane. The acid layer was divided into two portions for the spectrophotofluorometric assay of catecholamine and 5-hydroxytryptamine respectively. The catecholamines (adrenaline plus noradrenaline) were estimated in terms of noradrenaline in heart, spleen and small intestine. The amines were oxidized at *p*H 5 with iodine, the fluorescence then being developed in an alkaline ascorbate solution. This was measured at an activating wavelength of 400 mμ and an emission wavelength of 515 mμ. 5-Hydroxytryptamine fluorescence was read at *p*H 2 using an activating wavelength of 300 mμ and an emission wavelength of 540 mμ. This is essentially the method of Shore & Olin (1958) with the addition that 5-hydroxytryptamine is measured in the same sample (J. A. R. Mead, unpublished). The only further modification was that, after oxidation, the samples were subjected to ultra-violet irradiation for 7 min in order to speed up the development of maximum fluorescence and read immediately thereafter.

Isolated tissues. Pieces of rabbit intestine stimulated through the periarterial sympathetic nerve (Finkleman, 1930), and guinea-pig vas deferens stimulated through the hypogastric nerve (Boyd, Chang & Rand, 1960; Huković, 1960), were used, bathed in McEwen's solution (1956) aerated with oxygen plus 5% carbon dioxide. A section of rabbit ileum was set up in an isolated organ bath (vol. 100 ml.) at 37° C and the nerve stimulated for 30 sec every 4 min at a frequency of 50/sec from a constant voltage output. Alternate sections of ileum were set up under comparable conditions and either guanethidine added to the bath and stimulation continued until complete block was obtained or stimulation without the drug continued for the same length of time. Vasa deferentia from guinea-pigs were set up in a similar manner, one horn being stimulated in the presence of guanethidine until complete block was obtained, the other being stimulated for the same length of time in the absence of the drug. Tissues were removed from the bath, dried on filter paper, weighed and stored at −10° C until extracted for assay.

Blood pressure recording. Rats were anaesthetized with urethane (150 mg/100 g) intraperitoneally. The blood pressure was recorded from the carotid artery using a Condon manometer. Both femoral veins were cannulated with polythene tubing for the injection of drugs. The degree of sympathetic function present was measured by the pressor response to an intravenous injection of 20 μg of eserine (Varagić, 1955; Lešić & Varagić, 1961). Infusions of noradrenaline or dopamine were made at a rate of 0.05 ml./min over 20 min, through one femoral vein cannula, the other being used for the injection of eserine. The guanethidine and bretylium were injected subcutaneously.

Drugs. Bretylium was used as the tosylate, doses being in terms of the base. Doses of guanethidine were in terms of the sulphate. Eserine salicylate (with sodium metabisulphite 0.1 mg/ml. as preservative) was used; dose was of eserine base.

RESULTS

Amine depletion. Guanethidine greatly reduced the noradrenaline levels in spleen, heart and intestine. The effect was apparent at 1 hr, and reached a maximum of 80 to 90% at 6 to 18 hr. Some recovery had occurred by 48 hr (Fig. 1). Bretylium had no effect on noradrenaline levels in heart or spleen, but there was a rise in intestinal noradrenaline from 2 to 6 hr after injection and this had returned to normal by 18 hr.

The 5-hydroxytryptamine content of the spleen was not altered by either agent. No 5-hydroxytryptamine was detectable in the rat hearts. There was a transient

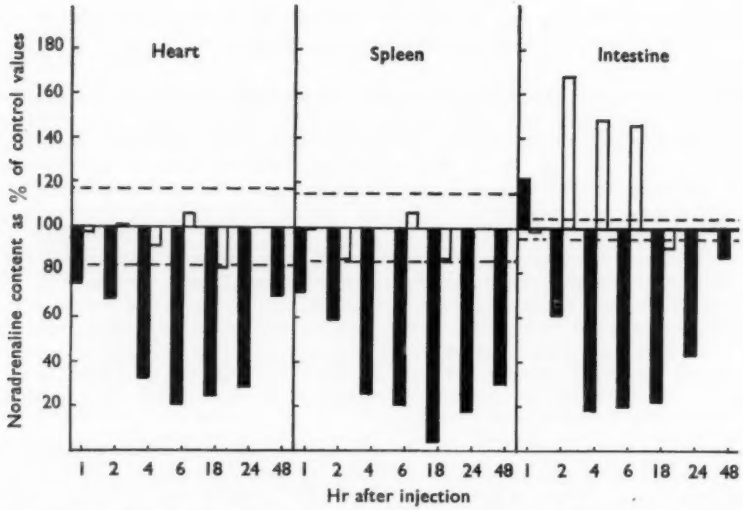

Fig. 1. Noradrenaline content of rat tissues after the subcutaneous injection of guanethidine 15 mg/kg (black columns) or bretylium 15 mg/kg (open columns). The dotted lines represent the standard error of the control levels.

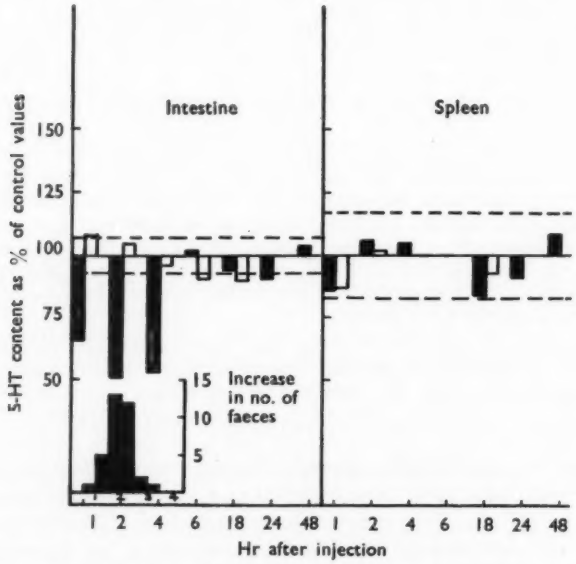

Fig. 2. 5-Hydroxytryptamine content of rat tissues after the subcutaneous injection of guanethidine 15 mg/kg (black columns) or bretylium 15 mg/kg (open columns). The dotted lines represent the standard error of the control levels. Inset: number of faeces passed by groups of rats after guanethidine, expressed as increase over the numbers passed by control groups. Abscissae: time in hr after injection.

depletion of intestinal 5-hydroxytryptamine after guanethidine which was maximal after 2 hr, returning to normal by 6 hr after injection. Diarrhoea was always a marked feature of the guanethidine-treated animals. When the number of faeces passed was counted at 30 min intervals, there was a large increase in the guanethidine-treated group compared with the other two groups (Fig. 2).

Sympathetic blockade. The pressor response to eserine was elicited repeatedly at 30 min intervals over a period of at least 6 hr. The height of the response was 26.3 ± 1.2 mm of mercury. It was confirmed that this response was present in rats adrenalectomized 2 weeks previously, but was eliminated in pithed rats. The injection of guanethidine resulted in an immediate fall of blood pressure, and subsequent injections of eserine were virtually without effect (Fig. 3). Some 6 hr

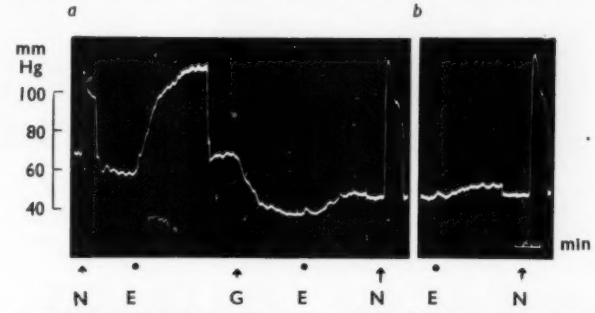

Fig. 3. Rat anaesthetized with urethane. Recording of blood pressure from carotid artery. The effect of guanethidine on the response to eserine. N=0.5 μg noradrenaline injected intravenously, E=20 μg eserine injected intravenously, G=guanethidine 15 mg/kg injected subcutaneously. Panel (*b*) shows the response 6 hr after G. Drum stopped after each response. E administered at 30 min intervals.

later the eserine response had not returned. When rats were treated 24 hr previously with guanethidine and the blood pressure recorded, the response was 10.8 ± 2.05 mm of mercury, that is, a 60% inhibition of the response to eserine. By 48 hr, however, the response was 24.6 ± 0.96 mm of mercury, which was not significantly different from the control response (Fig. 4). The pressor response to tetramethylammonium

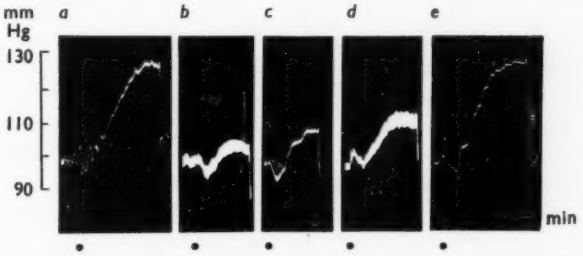

Fig. 4. Rat anaesthetized with urethane. Blood pressure recorded from the carotid artery. Typical responses to 20 μg eserine intravenously at the black dots, at various times after guanethidine 15 mg/kg subcutaneously. (*a*) Normal response, (*b*), (*c*), (*d*) and (*e*) respectively from rats injected with guanethidine 6, 18, 24 and 48 hr previously.

was not reduced by guanethidine and was sometimes potentiated. It was abolished by hexamethonium. Bretylium also caused a reduction of the response to eserine, but the onset was much slower and the degree of block was less.

Dopamine in doses up to 0.4 mg was ineffective in restoring the eserine response after blockade by guanethidine. However, 10 μg of noradrenaline sometimes increased the response, but its effectiveness was dependent on the time of infusion after the administration of guanethidine. Fig. 5 shows that noradrenaline when

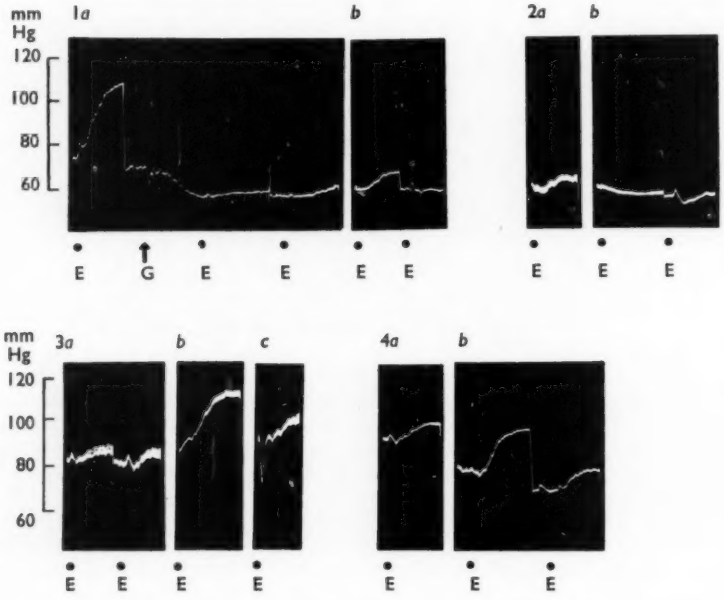

Fig. 5. Rat anaesthetized with urethane. Blood pressure recorded from the carotid artery. The effect of noradrenaline infusion on the response to eserine after guanethidine (G) 15 mg/kg subcutaneously. At E intravenous injection of 20 μg eserine. Between (*a*) and (*b*) 10 μg noradrenaline infused over 20 min. 1: 1·5 hr after G; 2: 3·5 hr after G; 3: 6 hr after G; and 4: 24 hr after G. Each number represents a different rat. 3(*c*) shows the response to eserine 30 min after 3(*b*).

infused 1 to 4 hr after guanethidine had little or no effect on the eserine response, but from 6 to 24 hr the response was restored. The infusion of noradrenaline had no effect on the response to eserine up to 18 hr after bretylium treatment.

Isolated tissues. Assay of segments of rabbit ileum or guinea-pig vas deferens in which guanethidine, in doses of 1 to 8 μg/ml., had produced complete block of the response to sympathetic nerve stimulation showed no depletion of noradrenaline content (Table 1): in fact, there was a slight increase. This increase was just significant ($P=0.05$) for the rabbit ileum.

TABLE 1

THE EFFECT OF GUANETHIDINE ON THE NORADRENALINE CONTENT OF RABBIT ILEUM (μG/G) AND GUINEA-PIG VAS DEFERENS (μG/HORN)

Tissue	Guanethidine	
	Absent	Present
Rabbit ileum	0·29±0·016	0·33±0·011
Guinea-pig vas deferens	1·17	1·46
	1·17	1·50
	1·47	1·72
	1·46	1·73

DISCUSSION

In the experiments described, an attempt has been made to test the hypothesis that the sympathetic block produced by guanethidine is related to a lack of tissue noradrenaline. Noradrenaline levels have been measured in heart, spleen and intestine and a marked and long-lasting depletion demonstrated. This is in agreement with the findings in rabbit and cat reported by Cass *et al.* (1960), and extends the observations reported for rats by Shepherd & Zimmermann (1959). These authors found a 50% depletion in heart and spleen 2 hr after 15 mg/kg guanethidine, but no effect after 24 hr. However, in their experiments the injection was made intraperitoneally and this may account for the much shorter duration of action. While these gross tissue levels are not necessarily a measure of changes in local concentration at the nerve terminals, a correlation between rate of measurable depletion and onset of sympathetic blockade would be a good indication that such a relationship existed.

Sympathetic function has been assessed by measuring the pressor response to a standard intravenous dose of eserine; this response has been shown by Lešić & Varagić (1961) to be mediated centrally. We have demonstrated that the response is reproducible over long periods, and that variation between animals is small. The response is unaltered by adrenalectomy and is absent in pithed rats. This confirms the findings of Lešić & Varagić and their conclusion that adrenal medullary discharge plays no part in the response. Guanethidine abolishes this response immediately, and in the anaesthetized rat the effect lasts at least up to 6 hr. There is evidence to suggest that the anaesthetic prolongs the primary blocking action of guanethidine (perhaps by slowing the rate of detoxication). In anaesthetized rats injected with guanethidine, there is no return of the eserine response over 6 hr. In rats given the drug less than 2 hr before the anaesthetic there is no response to eserine, but when guanethidine is administered more than 2 hr before the anaesthetic there is often a small response to the eserine and this does not increase over the period of the experiment. Since there is complete block of the response when the drug is given within 2 hr of the anaesthetic, comparison of the tissue levels measured in non-anaesthetized animals with the responses of the blood pressure during this period is valid.

Since Maxwell, Plummer, Schneider, Povalski & Daniel (1960) have demonstrated a short-lasting ganglion blocking action after guanethidine in the cat, the action of a ganglion stimulant drug, tetramethylammonium, has been tested before and during the first hour after guanethidine. Tetramethylammonium induced a rise of

blood pressure which was either unaffected or increased after guanethidine but which was abolished by hexamethonium. Since tetramethylammonium continued to stimulate the release of amines from the adrenal medulla in the presence of guanethidine, ganglion transmission appeared to be unaffected. The potentiation of the released amines probably compensates for the loss of activity at the post-ganglionic sympathetic sites.

It is clear, therefore, that the rate of onset of sympathetic block does not parallel the rate of depletion of peripheral noradrenaline. As previously discussed, this does not exclude a local but unmeasurable loss at the nerve ending. However, the results obtained using isolated tissues and those obtained by infusing noradrenaline into guanethidine-treated animals also indicate that there is no relationship between noradrenaline levels and the immediate blocking action of guanethidine. When block of the response to sympathetic nerve stimulation in the isolated tissues used was induced by guanethidine, there was no measurable loss of noradrenaline ; indeed, there was an increase, which for the rabbit ileum was statistically just significant. If the gross measurable changes are a slower reflection of small local changes it is unlikely that a measurable loss would be found within the time (8 to 44 min) that the tissues were exposed to guanethidine. If, in fact, the block were dependent on loss of transmitter, it is even more unlikely that a measurable increase would occur. It is interesting to note that, in the rat, intestinal noradrenaline was slightly increased at 1 hr after guanethidine injection, although by 2 hr depletion had occurred. Likewise after bretylium there was an increase in intestinal noradrenaline and this was sustained over 6 hr, bretylium having no depleting action. Schümann (1959) has shown in ox, sheep and dog that the principal catecholamine in the intestine is dopamine (95 to 100%). If this also applies to rabbit and rat it might provide an explanation for the increase in noradrenaline level seen in intestine. If the postulate that guanethidine and bretylium act by preventing the release or distribution of noradrenaline at the nerve ending is true, then treatment with these agents might be expected to result in a slight accumulation of noradrenaline. In tissues where noradrenaline or adrenaline are the principal catecholamines this increase might be masked, but in the intestine, where the proportion is normally very small, the increase might be revealed. Experiments are in progress to investigate this possibility.

Burn & Rand (1958, 1960) have shown that the response to certain sympathomimetic amines and to sympathetic stimulation, absent in the reserpine-treated animal, is restored by the infusion of noradrenaline or some of its precursors. Therefore the effect of infusions of dopamine or noradrenaline on the response to eserine after guanethidine or bretylium was investigated. The dose used was one which Burn & Rand had shown to be effective in restoring the response to tyramine in a pithed reserpine-treated rat. In our experiments it had little or no effect during the first few hours of guanethidine block, but, during the period when tissue noradrenaline levels were less than 25% of normal, noradrenaline infusion usually restored the eserine response to within limits normal for untreated rats. After bretylium, noradrenaline never increased the eserine response even in higher dosage. This again suggests that the primary block by guanethidine is unrelated to noradrenaline levels and that it may be similar to bretylium. It does, however, suggest

that the maintenance of the block may be due to a lack of noradrenaline. Burn (1961), in a review of adrenergic nerve function explaining the action of reserpine, bretylium and guanethidine, suggested that the hypotensive action of guanethidine was reserpine-like, that is, due to lack of noradrenaline. He also pointed out that guanethidine has a bretylium-like property. The evidence presented here suggests that this is the primary action of guanethidine and the reserpine-like action is secondary. The finding that dopamine, in doses 4 times those found by Burn & Rand to be effective in reserpine-treated rats, was ineffective in the guanethidine-treated rats is surprising. It may be due to the fact that our experiments were carried out in anaesthetized animals, whereas the results of these authors were obtained in pithed animals. Alternatively the use of eserine might have reduced the activity of dopamine dehydrogenase. Experiments are in progress to clarify these points.

One of the most common side-effects reported after clinical use of guanethidine is diarrhoea (Leishman, Matthews & Smith, 1959 ; Dollery, Emslie-Smith & Milne, 1960). Since 5-hydroxytryptamine has been implicated in the production of peristalsis both experimentally in animals (Bülbring & Lin, 1958) and in man (Lembeck, 1958), it was decided to investigate the action of both agents on 5-hydroxytryptamine levels. There was no alteration of spleen 5-hydroxytryptamine, but in the intestine guanethidine induced a 50% depletion after 2 hr. No 5-hydroxytryptamine has been detected in rat heart, but in rabbit and cat hearts no depletion of 5-hydroxytryptamine occurs after guanethidine (Cass *et al.*, 1960, and unpublished observations). The maximal depletion in the intestine coincided with the peak of peristaltic activity as measured by the number of faeces passed in unit time. Bretylium neither altered intestinal 5-hydroxytryptamine nor increased intestinal motility. This suggests a relationship between the liberated 5-hydroxytryptamine and the intestinal motility. It is not clear in this instance whether the 5-hydroxytryptamine released into the lumen of the intestine stimulates peristalsis as shown by Bülbring & Lin, or whether the guanethidine has a direct effect and the 5-hydroxytryptamine is released as a consequence of the increased activity. Reserpine induces marked diarrhoea, and it is one of the most common features of the carcinoid syndrome in which large amounts of 5-hydroxytryptamine are secreted from tumour tissue. These facts would indicate the first possibility as being the most likely. Since, however, the loss of 5-hydroxytryptamine after guanethidine seems to be specific to the intestine, the second possibility cannot be ruled out.

We wish to thank Dr M. J. Rand for assistance with the isolated tissue preparations and for helpful discussion and advice. Also Dr G. B. West for help in the preparation of the manuscript. We are grateful to Ciba Laboratories for supplies of guanethidine, and to Mr A. F. Green, of Burroughs Wellcome, for supplies of bretylium. One of us (T. L. B. S.) is in receipt of a grant from the Medical Research Council.

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ASSAY OF SUBSTANCE P ON GOLDFISH INTESTINE IN A MICROBATH

BY

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A technique is described for testing the effects of drugs on the intestine of goldfish in a microbath (0.05 ml.). This preparation may be used for the assay of substance P, which causes contractions of the muscle in doses as small as 1 m-u. Other substances in tissue extracts also act on this muscle, but the effects of most of these can be abolished by antagonists. Some tissues, however, contain an unknown substance which causes powerful contractions of the muscle and interferes with the assay of substance P. An attempt to demonstrate the release of substance P in the central nervous system was unsuccessful.

The object of the experiments recorded here was to find a sensitive and specific method for the assay of substance P. After preliminary experiments with the intestine of crayfish, frogs, pigeons and budgerigars, it was discovered that isolated pieces of the intestine of the goldfish contract in the presence of small amounts of substance P. The action of substance P on fish intestine has previously been described (Euler & Östlund, 1956). This paper describes a method of assay using this muscle in a microbath.

METHODS

Microbath. The microbath described by Gaddum & Stephenson (1958) has been modified in various ways for the present experiments (Fig. 1). The muscle is contained in a horizontal hole in a 2 cm cube of perspex at room temperature, through which the bath solution runs continuously at a rate of about 1 ml./min. Drugs are applied by stopping the flow by means of a relay and running a known volume of a solution containing the drug into one end of the bath. The volume of the bath is about 0.05 ml. and the volume of fluid containing the drug is also 0.05 ml. This volume is delivered from a small, clean glass tube into which it is sucked by withdrawing the plunger of an air-filled 0.25 ml. syringe by the appropriate amount.

The drug is left in contact with the intestine for a constant time (for example, 45 sec) and then the flow is restarted. The final concentration of the drug in the bath will be less than in the solution added, but results are reasonably constant. Previously drugs were driven in through a small hole. It has been found difficult to avoid direct mechanical effects on the record with this method, and drugs now are added to a vertical well from which they run slowly into the bath. The bathing fluid also runs through this vertical well and may accumulate to a height which depends on the rate of flow, the diameter of the hole connecting

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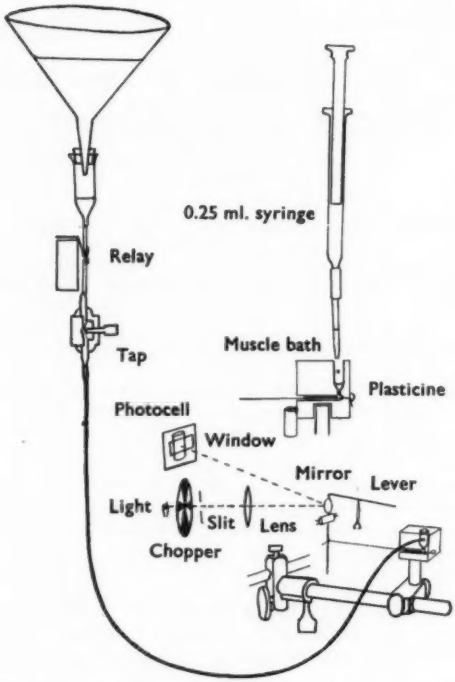

Fig. 1. Apparatus for perfusing the microbath (also shown in the inset). Diluted Locke solution runs from the funnel at a controlled rate of about 1 ml./min. At intervals the flow is stopped and 0.05 ml. of drug solution added to the well and allowed to run slowly into the microbath where it is left for a standard time.

the well with the bath and the rate of outflow from the bath. When the flow is stopped the fluid level in the well falls and the drug is not added until the well is empty. The bathing fluid accumulating in the well after the flow has been restarted will wash away any contamination from the previous application of a drug.

The bath fluid runs from a funnel through a small glass tube where the flow is measured by the formation of drops, and from there through a plastic tap (obtained from Townson & Mercer, Ltd., Croydon, England), designed for controlling slow flows. When the flow is stopped it is important that it should be completely stopped; this is achieved by the use of a relay which compresses a short piece of rubber tubing. All other parts of the system are made of glass or polythene—otherwise it is difficult to avoid complications due to the uptake and release of various substances (known or unknown) in the tubing.

Recording apparatus. In earlier work a torsion lever was used to record the contractions of the muscle. It has been found more satisfactory for the present purpose to use a simple isotonic lever. This is made of aluminium wire and consists of a horizontal arm, to which weights are attached, and a vertical arm, to which the thread from the muscle is attached. A small mirror is attached near the fulcrum and the liver is counterbalanced so that the tension depends solely on the attached weight. The tension in each muscle is adjusted and is generally about 100 mg weight. If it is too low, relaxation is poor and the tone continues to increase; if it is too high, drugs have little or no action.

A beam of light is chopped by a rotating sector and focused on the mirror. The image of a slit near the light is focused on a photocell. The output of the photocell is amplified by an AC amplifier and fed to an Elliott pen-recorder. The gain on the amplifier is adjusted so that full illumination of the photocell will give a full excursion of the pen-recorder. With a 3 cm photocell the movements of the muscle were magnified about 100 times. When greater magnification was desired a window was placed to reduce the effective height of the photocell to 1 or 0.5 cm and the gain increased accordingly. The greatest magnification was about 600 times. The object of the window was to prevent overloading of the pen recorder.

Isolated goldfish intestine. The goldfish (*Carassius auratus*) were generally 5 to 6 cm long. A piece of intestine about 5 mm long (unstretched) was removed from a point 2 to 5 cm from the anus. A fine terylene thread was tied round the intestine to attach it to the lever, and a second thread, attached to the other end of the intestine, was led through a very small hole at the bottom of the bath and fixed with a lump of plasticine.

Drugs used. In many of the experiments substance P was used in the form of a preparation prepared by Messrs. Hoffmann La Roche. This was compared with earlier standard preparations in this laboratory and in U. S. von Euler's laboratory and estimated to contain 75 Euler u./mg. It has been used in various laboratories as a provisional standard. In other experiments another preparation prepared in this laboratory was used. This preparation was estimated to contain 40 u./mg.

We are grateful to Messrs Sandoz for a sample of methysergide (UML 491, 1-methylysergic acid butanolamide) and to Messrs Parke, Davis & Co. for a sample of synthetic bradykinin.

Other drugs were obtained from the following sources: Dichloroisoprenaline—Eli Lilly; tryptamine hydrochloride—British Drug Houses; soyabean trypsin inhibitor—Light & Co.; chymotrypsin (crystallized) and trypsin (crystallized)—Armour & Co.; adenosine triphosphate—Sigma Chemical Co. The composition of the Locke solution in g/l. was the following: sodium chloride 9.0, potassium chloride 0.42, calcium chloride 0.24, glucose 2.0, sodium bicarbonate 0.2.

RESULTS

When the bath fluid was Locke solution the muscles generally gave repeated large contractions and were quite unsuitable for use. Occasionally they remained quiescent and appeared sensitive to substance P. Much time was therefore spent in trying to abolish these spontaneous movements. Various changes were made in the salt composition of the bath fluid, and drugs such as atropine, cocaine, alcohol, nitrates and adenosine were added, but none of these measures were effective. One evening when no drugs were available for trial, water was added to the Locke solution; the muscle contracted and then relaxed and remained quiescent, but sensitive to drugs. This procedure has been found regularly to provide good preparations; the muscle is first suspended in Locke solution, and after about 10 min, when spontaneous contractions start, the Locke solution is diluted by adding 40 ml. of distilled water for each 100 ml. of Locke solution. This causes a brief contraction followed by more regular spontaneous movements. After about 10 min this fluid is then replaced by Locke solution diluted with an equal volume of distilled water (1:2 Locke solution). This causes an immediate contraction followed by a slow relaxation; the spontaneous movements cease, but the muscle is still sensitive to drugs (Fig. 2). This stepwise dilution was found to produce more sensitive preparations than those obtained by omitting either of the preliminary stages.

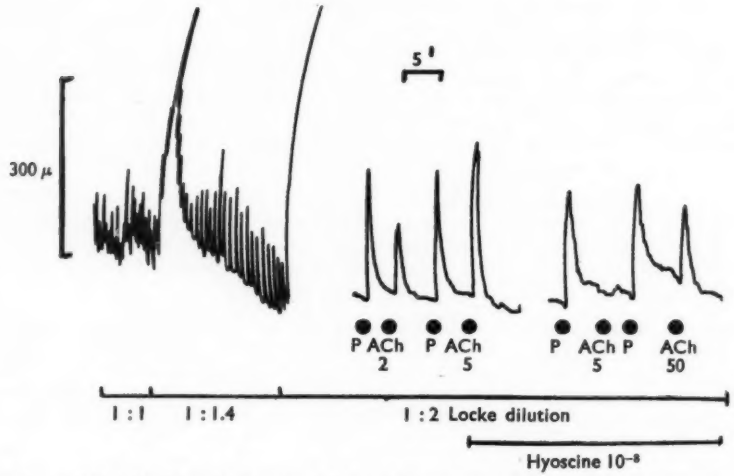

Fig. 2. Goldfish intestine in the microbath. Calibration shows actual shortening of the muscle. Dilution of the Locke solution caused disappearance of the spontaneous movements. P= substance P, 0.1 u./ml. ACh=acetylcholine (10^{-9}). Hyoscine (10^{-8}) antagonized acetylcholine but not substance P.

This effect of water appeared to be due to the hypotonicity of the solution. When the concentration of potassium chloride or calcium chloride was halved, the spontaneous contractions continued unchanged. When the concentration of sodium chloride was reduced to 50% or even 20% of the concentration in Locke solution, and the tonicity maintained with sucrose, the contractions also continued, but when it was reduced to 10% they ceased. Such preparations in which 90% of the sodium chloride had been replaced by sucrose remained quiescent and were, for a time, sensitive to drugs, but their sensitivity was not well maintained. These reactions have not been studied in detail. The tonicity of the solutions used appears to be much lower than the tonicity of goldfish blood ; there is some evidence that quiescent but sensitive preparations of other kinds of muscle can also be obtained by using hypotonic solutions (Szerb, 1961).

Since, with each administration of a drug, the entire bath fluid is replaced, small changes in the composition of this fluid may have an effect on the record. In order to know whether a reaction following the application of a small dose of drug is due to the drug itself, the bath fluid itself is applied as a control. In the absence of contamination, this should have little effect, but, with sensitive preparations, this effect may be appreciable.

The *p*H of the bath fluid was normally 6.9 to 7.1. A small increase (8.5) caused contraction of the muscle and a small decrease (5.5) relaxed it, but *p*H 2 caused contraction.

Substance P. Small doses of substance P caused a contraction starting 10 to 20 sec after its application, and disappearing in 1 to 2 min, when the time of

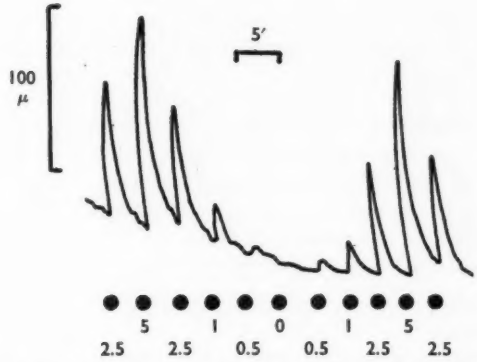

Fig. 3. As Fig. 2. Diluted Locke solution (1 : 2) containing hyoscine (10^{-8}), adenosine triphosphate (10^{-5}), and dichloroisoprenaline (5×10^{-8}). Contractions caused by substance P. Doses in milliunits contained in 0.05 ml.

contact with the tissue was 45 sec. The smallest concentration to have an effect significantly greater than control doses of the bathing fluid was generally 0.05 to 0.1 u./ml., but some preparations were more sensitive than this (see Fig. 3). The effect increased with the concentration over a 10-fold range of concentrations or more (Fig. 3) and application of the substance every 3 to 4 min did not lead to any appreciable decrease of the response.

The effect was fairly constant over a long period of time and satisfactory assays could be carried out. An extract of horse intestine was compared with the standard preparation in a (2+2) dose assay in the presence of atropine (10^{-8}) and adenosine diphosphate (10^{-5}). The concentrations of the standard were 0.25 and 0.5 u./ml. given every 4 min, each dose being repeated 4 times (Fig. 4). Since the volumes were 0.05 ml. the total amount of the standard used was 150 m-u.

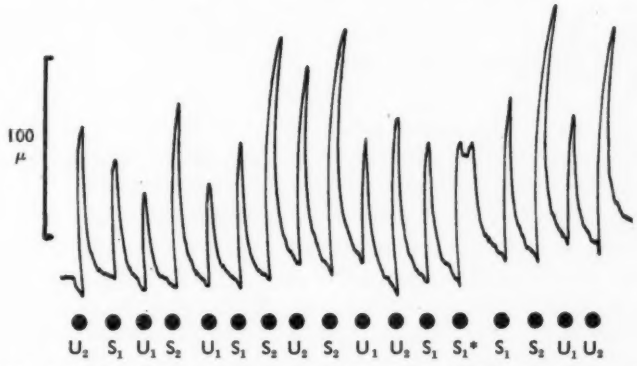

Fig. 4. As Fig. 2. Diluted Locke solution containing atropine (10^{-8}) and adenosine triphosphate (10^{-6}) (2+2) dose assay. S_1=0.25 u. standard substance P/ml. U_1=6.25 μg of an extract of horse intestine (P_3B). S_2 and U_2=double these doses. * Accidentally left in bath for 90 sec instead of 45 sec.

The analysis of variance showed that there was no significant deviation from parallelism, but the variance due to repetitions was highly significant, since the responses became larger during the test. The potency ratio was 76.4% and the index of precision ($\lambda = s/b$) was estimated as 0.0435. The corresponding value of Woolf's index of precision (Gaddum, 1953b) is 23, so that the test is reasonably precise. The extract of horse intestine was estimated in this experiment to contain 30 ± 3.4 ($P = 0.05$) u./mg. The mean results obtained by other methods were 42 (guinea-pig ileum), 37 (hen rectal caecum) and 31 (rat uterus). The differences between these results are of doubtful significance.

When substance P was left in the bath for longer times the contraction lasted 3 to 6 min and then disappeared, although the drug was not washed out (Fig. 5). The solution in the bath was then replaced with a fresh solution containing substance P. This caused another contraction so that the substance P in the bath must have

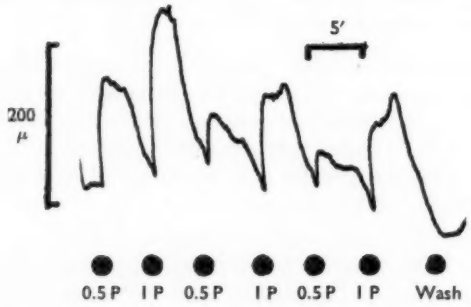

Fig. 5. As Fig. 2. Diluted Locke solution. No antagonist. Substance P was applied in alternate doses of 0.5 and 1.0 u./ml. without washing until the end.

been inactivated by the intestine. The second contraction was, however, smaller than the first, and successive contractions became smaller. This experiment suggests that there was also some tachyphylaxis.

Acetylcholine. Concentrations of acetylcholine between 10^{-9} and 10^{-8} caused a rapid contraction. This action was abolished by hyoscine or atropine (10^{-8}) so that the dose of acetylcholine had to be increased about 100 times to get similar effects (Fig. 2). This concentration of atropine reduced the effect of substance P; hyoscine was preferred since it appeared to be more specific.

Catecholamines. The muscle was inhibited by adrenaline (10^{-8}) or by noradrenaline (5×10^{-7}). The ratio of the concentrations of these two drugs causing equal effects was generally about 50 to 200. This ratio thus appears to be similar to that for the rat uterus and larger than that for the hen rectal caecum or for most other tests.

The effect of adrenaline was diminished or abolished when ephedrine (5×10^{-6}) was present in the bath, but the effect of noradrenaline was little, if at all, affected by this dose. Larger concentrations of ephedrine caused contractions of the muscle.

Ephedrine can thus be used to distinguish between effects due to adrenaline and effects due to noradrenaline, but cannot be used to exclude effects due to catecholamines when other substances are studied.

The effects of both adrenaline and noradrenaline were abolished by dichloroisoprenaline. When this substance was present in the bath in a concentration of 5×10^{-6} the relaxing effect of adrenaline was abolished and higher concentrations of adrenaline caused contraction. The relaxing effect of noradrenaline was also abolished by dichloroisoprenaline, but, in its presence, large doses of noradrenaline still caused relaxation (Fig. 6). Phenoxybenzamine (10^{-6}) did not affect the response to adrenaline or noradrenaline.

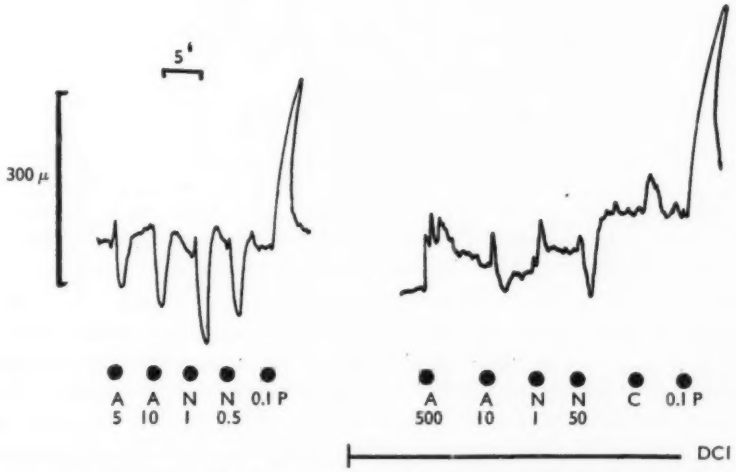

Fig. 6. As Fig. 2. Diluted Locke solution containing hyoscine (10^{-8}), adenosine triphosphate (10^{-5}) and methysergide (5×10^{-8}); A=adrenaline (ng/ml.); N=noradrenaline (μg/ml.); P=substance P u./ml.; C=control (no drug); DCI=dichloroisoprenaline, 5 μg/ml. (antagonizes A and N).

These results suggest that the inhibitory effect of the catecholamines depends on an action on β receptors. It is possible that high concentrations of ephedrine inhibit this action on β receptors. The effect of adrenaline was more easily inhibited than that of the much larger amounts of noradrenaline.

5-Hydroxytryptamine. The threshold concentration of 5-hydroxytryptamine causing contraction of the muscle was between 10^{-9} and 5×10^{-8}. A concentration of 1 to 2×10^{-8} was about equivalent to substance P 0.1 u./ml.

Tryptamine in larger doses also caused a contraction followed by relaxation in spite of the continued presence of tryptamine in the bath. Muscles which had been desensitized to tryptamine in this way were also insensitive to 5-hydroxytryptamine. This muscle thus resembled guinea-pig ileum in its response to these drugs

(Gaddum, 1953a). However, the relaxation of the goldfish intestine was often not complete in the presence of tryptamine and responses to other drugs became erratic.

Methysergide proved a more active and reliable antagonist than tryptamine. When it was present in the bath fluid in a concentration of 5×10^{-8} the muscle was specifically desensitized to 5-hydroxytryptamine so that the dose had to be increased 25 to 50 times (Fig. 7). This effect of methysergide developed in the course of about 1 hr.

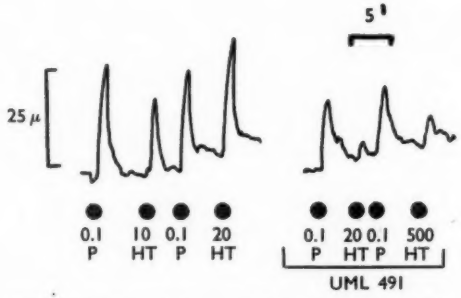

Fig. 7. As Fig. 2. Diluted Locke solution containing hyoscine (10^{-8}), adenosine triphosphate (10^{-5}) and dichloroisoprenaline (5×10^{-6}). HT = 5-hydroxytryptamine (10^{-9}); UML 491 = methysergide (5×10^{-8}). Antagonizes 5-hydroxytryptamine.

Histamine. The goldfish intestine is comparatively insensitive to histamine. A concentration of 10^{-6} sometimes caused a small contraction which was reversed by mepyramine. A concentration of 10^{-5} caused relaxation of the muscle. These small effects of large doses are unlikely to interfere with assays of other substances.

Adenosine compounds. The main effect of adenosine, adenosine 5-monophosphate and adenosine triphosphate was a contraction, sometimes following a brief relaxation. There was no marked and consistent difference between the effective concentrations of the three substances (about 10^{-6}), though adenosine triphosphate was generally less effective than the other two and its action was quicker.

The action of adenosine was abolished by atropine or hyoscine (10^{-8}). When adenosine (10^{-6}) was present in the bath fluid, the muscle was less sensitive to additional adenosine, but still sensitive to adenosine triphosphate.

The action of adenosine monophosphate was only reduced 5 times by hyoscine (10^{-8}). When adenosine triphosphate (10^{-5}) was also present in the bath fluid the effect was further reduced so that in the presence of both hyoscine and adenosine triphosphate the effective dose was increased 25 times.

The action of adenosine triphosphate (10^{-6}) was not affected by atropine. A concentration of 10^{-5} of adenosine triphosphate in the bath fluid caused an immediate powerful contraction followed by partial relaxation in the presence of the drug. The muscle was now 10 times less sensitive to additional doses of adenosine triphosphate (Fig. 8).

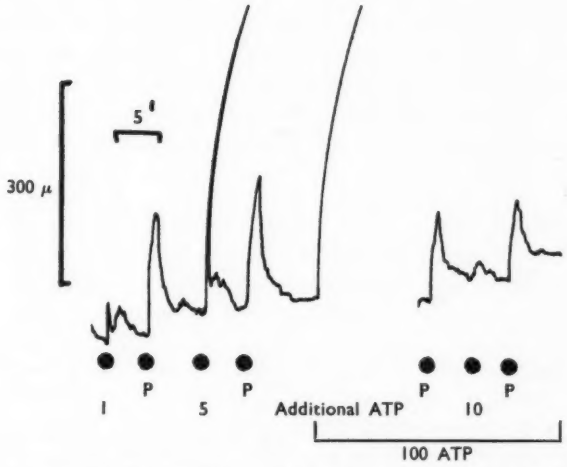

Fig. 8. As Fig. 2. Diluted Locke solution containing hyoscine (10^{-6}). P=substance P, 0.1 u./ml.; ATP=adenosine triphosphate 10^{-7}. Additional doses less effective in the presence of excess.

The presence of these concentrations of adenosine compounds did not diminish the effect of substance P. In some experiments it appeared to increase it.

Other polypeptides. Synthetic bradykinin has no action on the goldfish intestine except in high concentrations (2 to 10×10^{-6}). Oxytocin was without any effect in the concentrations tested (up to 0.1 u./ml.). Vasopressin caused a contraction when the concentration was 0.02 u./ml. and is thus the only one of these natural polypeptides which might possibly complicate assays of substance P.

Trypsin and chymotrypsin. Low concentrations of trypsin (0.2×10^{-6}) caused a contraction of the intestine, and this effect was antagonized by the soyabean trypsin inhibitor. In the experiment shown in Fig. 9 the intestine contracted in the presence of trypsin, but gave no response when a 250 times larger dose of trypsin was mixed with trypsin inhibitor before adding it to the bath. The response to trypsin reappeared within a few minutes when the inhibitor was removed from the bath.

The trypsin inhibitor had little or no effect on the response to bradykinin or substance P, so that it is possible to use it in experiments on the effect of trypsin on these substances which are present in tissue extracts. The extract is first incubated with trypsin, and the inhibitor is then added and the solution assayed for residual activity. The best method of carrying out this assay is probably to compare this incubated solution with another solution which is identical with it in all respects except that the inhibitor was added before incubation. The interpretation of such experiments is not complicated by the possible effect of the inhibitor on the gut, or by unspecific losses during incubation, or by other substances which may be present in the extract and alter the response of the intestine. It is known that trypsin destroys substance P, but not bradykinin, so that this test may be used to distinguish between the two substances. In one experiment, for example, incubation for 30 min

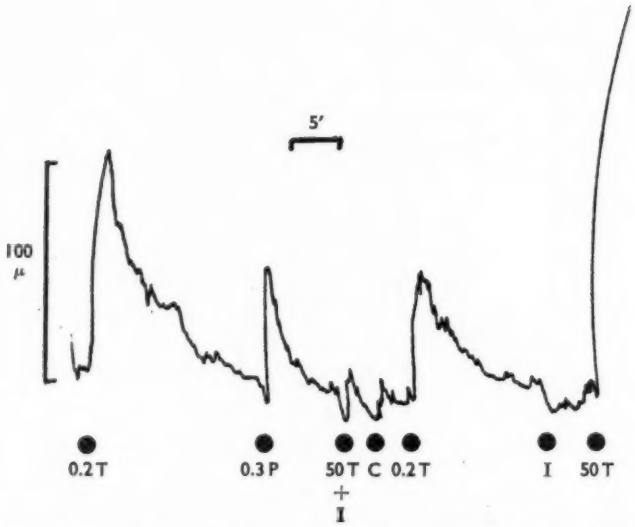

Fig. 9. As Fig. 2. Diluted Locke solution containing hyoscine (10^{-6}), adenosine triphosphate (10^{-5}) and methysergide (5×10^{-6}). P=substance P, u./ml.; T=trypsin, μg/ml.; I=soyabean trypsin inhibitor (5×10^{-4}). Antagonizes trypsin.

at 37° in the presence of trypsin (5×10^{-5}) almost completely abolished the effect of a preparation of substance P and only caused a slight loss of that of bradykinin. This slight loss might possibly have been due to a small trace of chymotrypsin in the preparation of trypsin, though there was no other evidence of this.

Chymotrypsin (5×10^{-6}) had no direct effect on the intestine, and reduced the effect of both substance P and bradykinin by 80% when incubated for 30 min at 37° C.

Unidentified substance. Simple watery extracts of guinea-pig brain contained some other unknown substance which caused a contraction of goldfish intestine even when much diluted. The effect of this substance overshadowed the effect of all the other substances known to be present in these extracts, so that a response was obtained with a dose corresponding to as little as 10 μg of fresh tissue. Similar effects were obtained with an extract of guinea-pig liver. The properties of this unknown substance are being studied in the hope of finding a method of separating it quantitatively from substance P.

Experiments with push-pull cannulae. The methods described in this paper are suitable for detecting active substances in the small volume of fluid which is obtained from a push-pull cannula (Gaddum, 1961). In this apparatus Locke solution runs through a fine needle into a tissue and is then collected through a tube surrounding the needle, so that a small volume of tissue is continually washed and substances released can be collected. Some experiments have been done with the object of detecting the release of substance P in the nuclei gracilis and cuneatus of a dog,

in the hope of obtaining evidence in favour of the theory of Lembeck (1953) that substance P is the chemical transmitter liberated by the first sensory neurone. In most of the experiments there was no evidence of the release of any substance acting on the goldfish intestine, and it is possible that the few positive results were artefacts, but the methods used will be briefly described.

The dogs were anaesthetized with allobarbitone (Dial) and the medulla oblongata was exposed by removing a small area of bone at the back of the skull. Push-pull cannulae were inserted through the pia to a depth of 1 to 5 mm at various points both above and below the obex and at different distances from the midline. Locke solution was run through the cannulae at a rate of 0.5 to 2 ml. in 10 min and collected in a centrifuge tube standing in ice. The samples collected sometimes contained a visible trace of blood, but were usually quite clear. These samples were diluted with an equal volume of a diluting fluid, which consisted of distilled water, containing double concentrations of any antagonists which were present in the fluid bathing the intestine. It was usually possible in this way to avoid artefacts, so that fluids which had run through the cannula before it was placed in the tissue had no action on the intestine. In most experiments the diluting fluid contained hyoscine 2×10^{-8} and dichloroisoprenaline 10^{-5}. In some of the earlier experiments stimulation of sensory nerves in the fore and hind limbs appeared to release small quantities of a substance which caused contraction of the goldfish intestine, but this substance differed from substance P in the fact that it was unstable and acetone soluble, and in the last 6 experiments no activity was detected at all.

DISCUSSION

The approximate concentrations of various substances which cause a small effect in 4 tests for substance P are shown in Table 1. The sensitivity of these tissues varies widely and the figures only give a rough indication of average values. All 4 tests are sensitive to about the same concentration of substance P, though the

TABLE 1

APPROXIMATE CONCENTRATIONS (NG/ML.) OF VARIOUS SUBSTANCES REQUIRED TO PRODUCE SMALL EFFECTS ON DIFFERENT TISSUES

Brackets denote inhibition

	Rat uterus	Guinea-pig ileum	Hen rectal caecum	Goldfish intestine
Substance P (u./ml.):	0·1–0·5	0·1–0·5	0·1–0·5	0·05–0·1
Polypeptides:				
Bradykinin	0·1	1	>10,000	2,000–10,000
Angiotensin	0·2	1	100	$>10^4$
Oxytocin	0·2	>500	>400	>200
Vasopressin	4	500	360	40
Other substances:				
Acetylcholine	250	10	5	1–10
Histamine	(10^4)	1	10	1,000
5-Hydroxytryptamine	10	40	100–1,000	10–50
Adrenaline	(0·05)	(10)	(1)	(10)
Noradrenaline	(10)	(20)	(40)	(500)
Adenosine triphosphate	1,000	(100)	(400)	1,000

goldfish intestine is slightly more sensitive and has the advantage that it can easily be used in a microbath with a volume of 0.05 ml. There is no other test which will detect 1 m-u. of substance P. This corresponds to about 30 pg of the preparation described by Franz, Boissonnas & Stürmer (1961).

The data given in Table 1 for other substances are taken from various sources. The figures for the rat uterus are mostly from Gaddum, Peart & Vogt (1949). The doses of bradykinin for rat uterus and guinea-pig ileum are from Elliott, Horton & Lewis (1960); in our experience the guinea-pig ileum is about 10 times less sensitive than this. The figures for angiotensin on these two tissues are from Gross & Turrian (1960). The figures for hen rectal caecum were mostly obtained by Cleugh (1961). Using pure synthetic bradykinin the dose of bradykinin was about 10,000 ng/ml. It may be calculated from the data of Van Arman & Miller (1961), who used an impure preparation made from trypsin, that the hen rectal caecum was about 1,000 times more sensitive than this. This calculation agrees with other evidence which suggests that partially purified preparations of bradykinin may contain a second active substance with an action on hen rectal caecum.

The goldfish intestine is comparatively insensitive to histamine and to the other polypeptides likely to be present in tissue extracts. If suitable antagonists are added, it may be made insensitive to other substances (Table 2), but it will be seen

TABLE 2

CONCENTRATIONS (μG/ML.) OF VARIOUS SUBSTANCES REQUIRED TO PRODUCE SMALL EFFECTS ON THE GOLDFISH INTESTINE IN THE ABSENCE AND PRESENCE OF ANTAGONISTS

Concentrations of antagonists in μg/ml. Dichloroisoprenaline=D.C.I.
Adenosine triphosphate=A.T.P.

Agonist			Antagonist	
	Without antagonist	With antagonist		
Acetylcholine	0·01	1	Hyoscine	0·01
5-Hydroxytryptamine	0·015	0·4	Methysergide	0·05
Adrenaline	0·01	0·05–0·1	D.C.I.	5
Noradrenaline	0·5	100	D.C.I.	5
Adenosine	2	>1,000	Hyoscine	0·01
Adenosine monophosphate	2	50	Hyoscine +A.T.P.	0·01 10
Adenosine triphosphate	0·2–1	10	A.T.P.	10

that the effects of all these antagonists can be surmounted by increasing the dose of the agonist. In the presence of the antagonists these substances are unlikely to affect the results unless they are present in very high concentrations, but it would clearly be unwise to conclude that any effects were due to substance P without confirmatory evidence.

The possibility that the effects are due to acetylcholine can sometimes be excluded by estimating the concentration of this substance by some other method such as that based on the leech (Szerb, 1961). Another way is to compare unknown solutions on the goldfish intestine with standard substance P, both in the absence and presence of hyoscine. Similar tests may be used to exclude 5-hydroxytryptamine or adenosine compounds.

The active substance may also be tested for solubility and stability and by paper chromatography or electrophoresis in comparison with authentic substance P. It should be destroyed by trypsin, and a test for this is shown in Fig. 9.

When extracts of guinea-pig brain were tested, the apparent concentration of substance P measured with the goldfish was so large that it should have been easily detected by various other methods. It is clear that this effect was not due to substance P, but to some other substance. It will not be possible to get reliable estimates of substance P with the goldfish until the effects of this unknown substance can be excluded.

We are indebted to J. Lucas, who did the first experiments with the intestines of various species of animal and gave patient help later.

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DIURESIS IN RATS: EFFECTS OF SYMPATHOMIMETIC AND SYMPATHETIC BLOCKING AGENTS

BY

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Dopamine and tyramine possess diuretic properties resembling but weaker than those of noradrenaline in the rat. These three amines produce a relatively smaller loss of sodium than does adrenaline. Bretylium shows a diuretic action which is apparently associated with its sympathomimetic properties and which is antagonized by phenoxybenzamine. However, bretylium causes a relatively greater loss of potassium and chloride than do the sympathomimetic amines. A slight antidiuretic action is shown by bretylium after its diuretic effect has subsided and a similar effect is produced by BW 172C58, which has relatively weak sympathomimetic properties. In contrast to these adrenergic neurone blocking agents, phenoxybenzamine and a ganglion blocking agent (pentacynium) show powerful antidiuretic effects. These observations are consistent with the view that the adrenal medulla has an important role in facilitating water diuresis in rats.

Adrenaline and noradrenaline cause diuresis in the rat (Dexter & Stoner, 1952). It was therefore interesting to investigate the effects of dopamine, the noradrenaline precursor, and tyramine, which releases catechol amines (Burn & Rand, 1958; Lockett & Eakins, 1960; Schümann, 1961). Bretylium, a compound with early sympathomimetic action, and *N*-[2-(4-benzoyl-2,6-dimethylphenoxy)ethyl]-*N*,*N*,*N*-trimethylammonium methyl sulphate (BW 172C58), a compound with relatively little sympathomimetic effect (Boura & Green, 1959; Boura, Coker, Copp, Duncombe, Elphick, Green & McCoubrey, 1960), were compared with these amines.

However, the main and more persistent action of bretylium and BW 172C58 is to depress adrenergic nerve function without inhibiting the activity of the adrenal medulla (Boura & Green, 1959; Boura *et al.*, 1960), and it is therefore of further interest to compare their effects on kidney function in the rat with those of the ganglion blocking agent pentacynium (Green, 1956) and the adrenaline antagonist phenoxybenzamine.

METHODS

Male Wistar rats weighing 100 to 200 g from a closed colony were used. For 18 hr before testing they were allowed no solid food but water *ad lib*. They were divided at random into groups of 5, received water or saline by stomach tube and were placed in metabolism cages. Except where stated the drugs were administered subcutaneously immediately after the water or saline load. The drugs were given in saline, 0.5 ml./100 g rat, and a similar volume of saline was given to controls. Urine volumes were recorded at 30 min intervals usually for 3 hr. They have been expressed as percentages of the total volumes of fluid administered.

In diuretic tests, 0.9% sodium chloride solution at 37° C, 1 ml./100 g, was given by stomach tube. In antidiuretic tests water at 37° C, 5 ml./100 g, was given orally. At least three separate tests on groups of 5 rats at each dose level were combined to obtain the mean values shown in the tables.

Urine sodium and potassium concentrations were determined by flame photometry and chloride by Volhard's method.

The effect of phenoxybenzamine and pentacynium on the absorption of water from the alimentary tract and on the retention of urine was examined in groups of 6 fasted rats. Phenoxybenzamine was given 3 hr before and pentacynium immediately after giving a water load of 5 ml./100 g. Controls received saline 0.9% sodium chloride solution, 0.5 ml./100 g, at the same time as the water load. The rats were placed in individual metabolism cages. Each rat was killed 1 hr later and the volume of urine in the bladder was measured with a tuberculin syringe. The weight with contents of stomach, small intestine and caecum of each rat was also determined. The effect of bretylium on the urine content of the bladder was determined 1 hr after administering the drug at the same time as a 0.9% sodium chloride solution load of 1 ml./100 g orally in groups of 6 fasted rats.

Bretylium bromide and bretylium tosylate (" Darenthin ") were used, but doses are expressed as bromide. BW 172C58 was given as bromide or methyl sulphate ; amounts refer to the bromide equivalent. Doses of pentacynium *bis* (methyl sulphate), phenoxybenzamine hydrochloride, tyramine hydrochloride, dopamine hydrochloride are those of the salts used, but doses of (−)-adrenaline and (−)-noradrenaline are in terms of base. Chlorothiazide was dissolved in 0.9% sodium chloride solution by adding a minimum of sodium hydroxide.

Comparisons of differences between pairs of groups of animals were assessed by the exact method of Fisher, the 5% level being used as the criterion of significance.

RESULTS

Diuresis

The volumes of urine excreted by saline-treated rats at intervals after the administration of adrenaline, noradrenaline, dopamine, tyramine, bretylium and BW 172C58 are shown in Fig. 1. Each of the catechol amines tested showed powerful diuretic actions, noradrenaline being the most active and dopamine easily the least active. The effects of noradrenaline and dopamine were more rapid in onset than those of adrenaline. Tyramine was slightly less active than dopamine. A powerful diuretic action was also shown by bretylium but not by BW 172C58.

The effects of bretylium, adrenaline and noradrenaline on electrolyte excretion are compared in Fig. 2. Sodium concentration was slightly but significantly increased by diuretic doses of bretylium and adrenaline except where the latter was given at very high dosage (4 mg/kg). In contrast, all doses of noradrenaline (except 0.25 mg/kg) caused a significant fall in sodium concentration. The total output of sodium was increased by diuretic doses of all three drugs, but was least with noradrenaline. Potassium concentration was lowered by all three compounds to extents that increased with the dosage, the change being greatest with adrenaline and least with bretylium. Total potassium output was increased significantly only by bretylium (for all doses examined $P =$ or < 0.01). The mean chloride concentration, 155 mEq/l. (s.e. ± 7.6) in controls, was not appreciably changed by any of the doses of bretylium nor by 0.25 or 0.5 mg/kg adrenaline but was reduced to 110 ± 4.5 mEq/l. by 4 mg/kg adrenaline. All the doses of noradrenaline tested significantly lowered the chloride concentration, values of 120 ± 13 and 91 ± 12

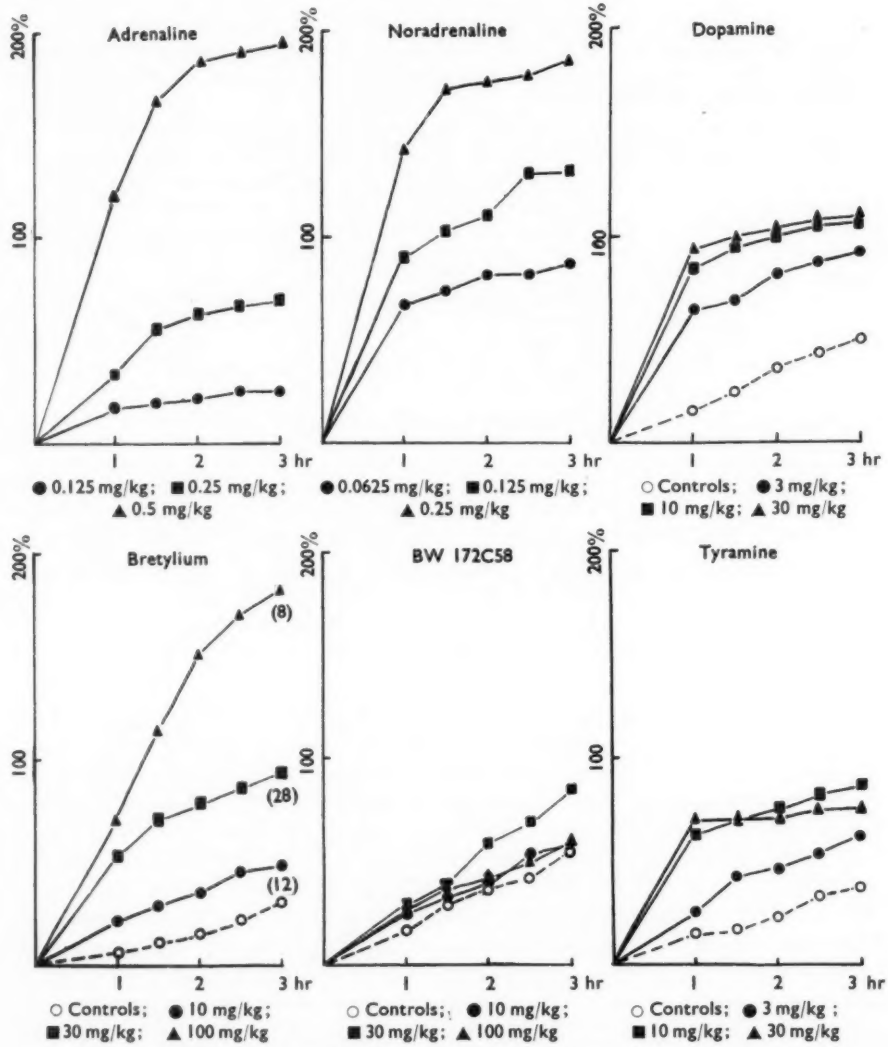

Fig. 1. Diuretic effects in rats. Ordinates: urine volume as a % of the total fluid load. Abscissa: hr after giving drug and saline load. The control shown under bretylium is from 26 experiments; those for dopamine, tyramine and BW 172C58 were concomitant. The numbers of groups of 5 rats given each dose level of bretylium are shown in brackets. All other treatments were given to 3 groups of 5 rats.

being found after the doses of 0.0625 and 2 mg/kg respectively. The mean ratio of the sum of the sodium and potassium concentration to the chloride concentration in mEq/l. was about 1.6 in controls. Similar ratios were found in bretylium-treated rats but significantly lower values occurred after adrenaline and noradren-

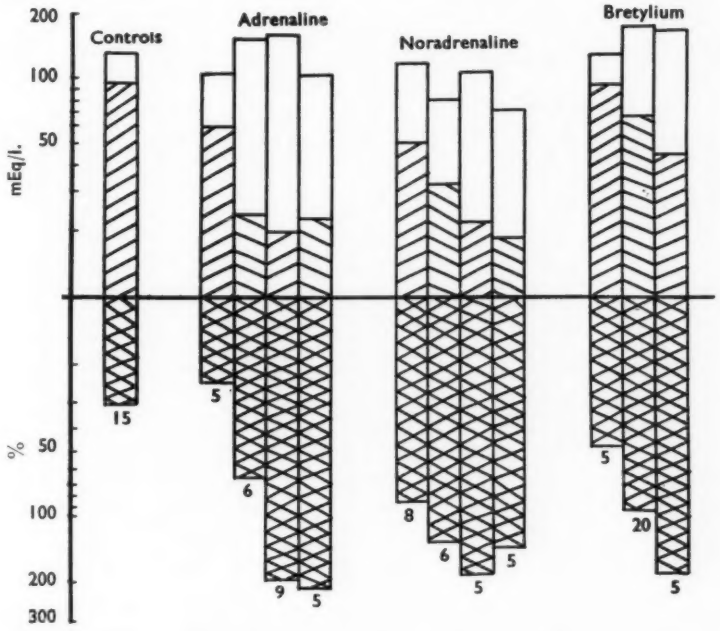

Fig. 2. Diuresis in rats. Electrolyte concentrations and total urine volumes excreted 3 hr following drug administration. The lower limits of the cross-hatched columns below the base line indicate urine volumes as a % of the total saline load. The heights above the base line of the clear and shaded portions of the columns represent sodium and potassium concentration respectively, in mEq/l. The logarithmic scales are arranged so that the total sodium and potassium excretions are proportional to the combined spans of the columns. The overall mean standard deviations of the % urine volumes, sodium concentrations and potassium concentrations were of the order of ± 25, ± 36 and ± 16 respectively. The number of tests at each dose level is shown below the columns. Columns from left to right: controls; adrenaline, 0.125, 0.25, 0.5 and 4.0 mg/kg; noradrenaline, 0.0625, 0.125, 0.25 and 2 mg/kg; bretylium, 10, 30 and 100 mg/kg.

aline. Thus the mean ratios were 1.24, 1.35 and 1.16 after 0.25, 0.5 and 4 mg/kg adrenaline respectively, and 1.14 and 0.90 after 0.0625 and 2.0 mg/kg noradrenaline respectively.

A low dose of adrenaline (0.125 mg/kg) tended to reduce the urine output and significantly lowered sodium and potassium concentrations. These results in the rats loaded with 0.9% sodium chloride solution are in keeping with those in the water-loaded rat as reported by Botting & Lockett (1961) and Botting, Farmer & Lockett (1961).

A comparison between the effects on electrolyte excretion of bretylium, adrenaline, dopamine and tyramine in another series of similar experiments is shown in Fig. 3. The sodium:potassium balance was slightly different in the control rats of this series. Nevertheless the changes produced by bretylium and adrenaline were similar to those in the first series. Bretylium was again the only compound which signifi-

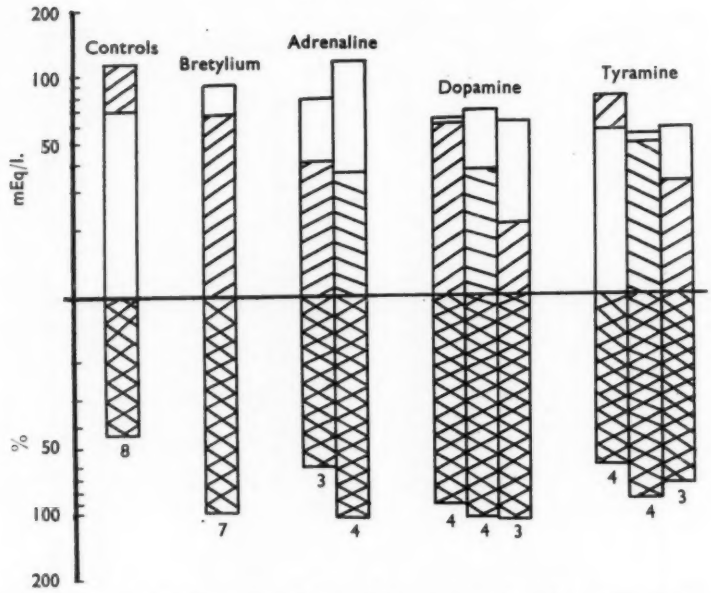

Fig. 3. As Fig. 2, but columns from left to right: controls; bretylium, 30 mg/kg; adrenaline, 0·25 and 0·5 mg/kg; dopamine, 3, 10 and 30 mg/kg; tyramine, 3, 10 and 30 mg/kg.

cantly increased potassium output. Dopamine and tyramine at submaximally effective doses (3 to 10 mg/kg) showed a relatively greater sodium-retaining effect and caused a relatively smaller change in the sodium:potassium ratio than did adrenaline. In these respects the effects of dopamine and tyramine resembled those of noradrenaline.

TABLE 1

ANTAGONISM OF THE DIURETIC EFFECTS OF ADRENALINE AND NORADRENALINE BY VARIOUS DOSES OF PHENOXYBENZAMINE GIVEN 3 HR PREVIOUSLY

	2 hr urine volume (% of fluid load)	
Phenoxybenzamine mg/kg	Adrenaline, 0·5 mg/kg	Noradrenaline, 0·25 mg/kg
0	113	173
0·0125	67	140
0·125	22	91
1·25	6	29

The diuretic effects of adrenaline and noradrenaline could be greatly reduced by treating the rats with phenoxybenzamine 3 hr earlier, the degree of antagonism being proportional to the dose of phenoxybenzamine (Table 1). Pretreatment with phenoxybenzamine 3 hr previously also reduced the diuretic response to bretylium but not to chlorothiazide (Table 2).

Just as bretylium potentiates many other effects of adrenaline and noradrenaline (Boura & Green, 1959), so also it increased the diuretic action of these substances. In these experiments sufficient time (4 hr) was allowed for the sympathomimetic

TABLE 2

EFFECT OF PHENOXYBENZAMINE ON THE DIURETIC EFFECT OF CHLOROTHIAZIDE AND BRETYLIUM

Phenoxybenzamine mg/kg	2 hr urine volume (% of fluid load)		
	Bretylium 30 mg/kg	Bretylium 100 mg/kg	Chlorothiazide 5 mg/kg
0	130	172	118
0·1	76	63	103

TABLE 3

DIURETIC EFFECTS IN RATS TREATED WITH BRETYLIUM

Bretylium was given 4 hr before the diuretic drugs

Bretylium mg/kg	2 hr urine volumes (% of fluid load)			
	Adrenaline 0·25 mg/kg	Adrenaline 0·5 mg/kg	Noradrenaline 0·0625 mg/kg	Chlorothiazide 5 mg/kg
0	30	161	104	107
30	105	227	127	96

action of bretylium to decline, before giving the catechol amines. Whereas the increase in the effect of adrenaline shown in Table 3 is large, that for noradrenaline is less prominent, but nevertheless significant at the 5% level. Under similar conditions bretylium tended to reduce the diuretic action of chlorothiazide.

Antidiuresis

When 30 mg/kg bretylium was given at the same time as a large water load, it increased the rate of diuresis. However, since an antidiuretic action might occur once the sympathomimetic diuretic action had subsided, water loads were given 2 or 4 hr after administering bretylium. An attempt was made to ensure that the bretylium-treated and control animals were in a similar state of hydration at the time of water loading, by giving orally, at the same time as the bretylium, a volume of 0.9% sodium chloride solution roughly equal to the urine excretion that would occur as a result of its diuretic action. These volumes of 0.9% sodium chloride solution given to the 30 mg/kg, 100 mg/kg and control groups were 1, 3 and 0.25 ml./100 g respectively. Table 4 shows that 30 mg/kg bretylium given 2 hr before the water load, or 30 to 100 mg/kg given 4 hr before the water, slightly retarded the onset of diuresis. No antidiuretic action was seen 2 hr after 100 mg/kg bretylium, presumably because the masking diuretic action of this dose was more persistent. BW 172C58, which has little sympathomimetic action, had a very weak antidiuretic effect when given at the same time as the oral water load. No antidiuretic action was seen in 3 other experiments where doses of 30 or 100 mg/kg BW 172C58 were given 2 hr before the water load. In contrast to the adrenergic neurone blocking agents, the ganglion blocking agent pentacynium and the adrenaline antagonist phenoxybenzamine showed powerful antidiuretic properties.

Effect on absorption of water and retention of urine. Large doses of phenoxybenzamine (12.8 mg/kg) had no effect and pentacynium (5 mg/kg) had only a small delaying effect on the absorption of water from stomach and small intestine, so far as

TABLE 4

ANTIDIURETIC EFFECTS OF BRETYLIUM, BW 172C58, PENTACYNIUM AND PHENOXYBENZAMINE GIVEN AT VARIOUS TIME INTERVALS BEFORE A WATER LOAD

Urine volumes at 1 and 2 hr after the water load are shown as % of total fluid load

Treatment		Interval	Urine volumes	
Drug	mg/kg	hr	1 hr	2 hr
Bretylium	30	2	16	56
Bretylium	100		41	67
Controls			33	75
Bretylium	30	4	16	80
Bretylium	100		14	62
Controls			28	73
BW 172C58	30	0	29	69
BW 172C58	100		23	43
Controls			22	71
Pentacynium	0·625	0	26	59
Pentacynium	1·25		11	34
Pentacynium	2·5		7	24
Pentacynium	5·0		3	13
Pentacynium	10·0		2	6
Controls			47	85
Phenoxybenzamine	0·05	3	30	74
Phenoxybenzamine	0·2		20	71
Phenoxybenzamine	0·8		15	66
Phenoxybenzamine	3·2		8	52
Phenoxybenzamine	12·8		3	22
Controls			44	79

could be ascertained by weighing these organs 1 hr after giving a water load. The alimentary tract of pentacynium-treated rats contained significantly more water than controls, but they had absorbed about 70% (5 ml.) of the water load. Thus this difference can only partly account for the antidiuretic effect of the compound. The volumes of urine found in the bladders of the rats given phenoxybenzamine and pentacynium tended to be less than those in the controls (mean 0.27 ml.). The antidiuretic action of these compounds cannot therefore be due to retention of urine. The volumes of urine found in the bladders of rats examined 1 hr after a saline load (1 ml./100 g) were similar in bretylium-treated rats and controls. Hence the diuretic effect of the drug cannot be attributed to any effect on the bladder.

DISCUSSION

Earlier reports that adrenaline and noradrenaline exhibit powerful diuretic effects in the rat are confirmed and it is further shown that in rats loaded with 0.9% sodium chloride solution noradrenaline causes a relatively smaller loss of sodium and chloride than does adrenaline. Low doses of adrenaline were found by Botting *et al.* (1961) to produce antidiuresis with retention of sodium and potassium in water-loaded rats as in our experiments on saline-loaded rats. Quantitative differences between the effects of adrenaline and noradrenaline in increasing water, sodium, and chloride output and decreasing urinary potassium concentration have been reported by Giere (1954), but these results in water-loaded rats are difficult to equate with our results in saline-loaded rats. Diuresis was also produced by dopamine and tyramine in the rat, and, in so far as they caused a relatively smaller

loss of sodium than did adrenaline, their action resembles that of noradrenaline. Just as dopamine is a weaker pressor agent than noradrenaline so also is its diuretic activity less. The diuretic action of tyramine is in keeping with its known releasing effect on noradrenaline stores (Burn & Rand, 1958; Lockett & Eakins, 1960; Schümann, 1961).

Bretylium also causes diuresis in rats. This may be another manifestation of its sympathomimetic action which is apparently due to catechol amine release in the rat (Gillis, 1960) as in other species (Boura & Green, 1959), since the diuresis like that of adrenaline and noradrenaline is readily inhibited by phenoxybenzamine. Moreover, BW 172C58, an adrenergic neurone blocking agent with relatively weak sympathomimetic properties (Boura *et al.*, 1960), showed very little diuretic action in rats. However, the relatively greater loss of potassium and chloride found in rats given bretylium, as compared with that in rats given adrenaline, noradrenaline or dopamine, indicates that the change in electrolyte excretion is not entirely due to bretylium releasing any one of these amines. Nevertheless, the possibility of the effect of bretylium being due to release of a combination of these amines and perhaps also isoprenaline is not excluded. Alternatively, the difference between the change in electrolyte composition seen with bretylium and that with the catechol amines studied, or with tyramine, could be related to the adrenergic neurone blocking effect of bretylium. This possibility deserves further investigation. Despite the increased potassium excretion seen with bretylium, no significant change was seen in the serum level of potassium when groups of 6 rats were examined 20 and 60 min after 100 mg/kg bretylium. Tissue levels have still to be examined.

Eversole, Giere & Rock (1952) found that noradrenaline caused an increase of glomerular filtration rate and also an apparent decrease in tubular reabsorption of water. Glomerular filtration rate is also likely to increase with diuretic amounts of adrenaline and bretylium. However, the changes in sodium: potassium ratios following the various compounds may indicate effects on the renal tubules. The rise of chloride, relative to sodium and potassium, found in the urine of rats treated with adrenaline or noradrenaline suggests that the amines may inhibit the tubular reabsorption of chloride, particularly as an analogous effect was not produced by bretylium. Botting *et al.* (1961) found that low doses of adrenaline and isoprenaline showed antidiuretic effects without reducing glomerular filtration rate, indicating an effect on renal tubular function.

Phenoxybenzamine and pentacynium showed powerful antidiuretic effects in the rat, and the action of phenoxybenzamine is similar to that described for Dibenamine (Horres, Eversole & Rock, 1950). The effective doses of phenoxybenzamine are similar to those that reduce the diuretic action of injected adrenaline and noradrenaline. The antidiuretic action of pentacynium occurs with doses comparable to those known to block autonomic ganglia and the mechanism of activation of the adrenal medulla in the rat (Green, 1956). Hence the antidiuretic effects of phenoxybenzamine and pentacynium can be related to their effects on sympathetic nerve function or on the adrenal medulla or both. In contrast, bretylium and BW 172C58 do not show powerful antidiuretic effects, despite ability to abolish adrenergic nerve function (at least in other species), which might be related to their lack of effect on

the adrenal medulla (Boura & Green, 1959 ; Boura *et al.*, 1960). Such an explanation would conform with other evidence indicating that the adrenal medulla plays a major role in bringing about water diuresis in rats (Dexter & Stoner, 1952). An alternative explanation of the lesser antidiuretic actions of bretylium and BW 172C58 would be that the fall of sympathetic tone produced by these compounds was less or was offset by their residual or inherent sympathomimetic actions. Though sympathetic blockade might cause antidiuresis by a reduction of glomerular filtration rate, this cannot at present be related to depression of systemic blood pressure. It has been reported that the blood pressure of unanaesthetized rats is increased by ganglion blocking agents and phentolamine (van Proosdij-Hartzema & de Jongh, 1955) and decreased by bretylium (van Proosdij-Hartzema, personal communication), even though the opposite effects are prominent in the anaesthetized rat. Further, the possibility is still open that sympathetic blockade may affect the activity of renal tubules, producing effects opposite from those of the sympathomimetic amines.

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EFFECT OF CHLORPROMAZINE ON SEPTAL HYPERACTIVITY IN THE RAT

BY

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The effect of chlorpromazine on the overt emotional activity of a hybrid strain of rats has been measured using an altered "emotionality rating scale." It has been found that 8 mg/kg of chlorpromazine intraperitoneally reduced the activity of rats with the septal nuclei destroyed, normal rats and control rats to the same emotional rating. This effect has been related to the postulated interrelationship of the septal area and the amygdaloid complex of nuclei. It seemed that impulses traversing the septal nuclei might be re-routed, so that destruction of the septal area need not necessarily cause total abolition of "septal-function."

It has been suggested by Olds & Travis (1960) that chlorpromazine can inhibit a forebrain system, while leaving intact the functions of a midbrain system. This is compatible with the hypothesis that chlorpromazine activity is localized within the limbic lobe.

A marked increase in emotional behaviour of animals after experimental ablation of the septal region of the forebrain has been reported (Brady & Nauta, 1953; King, 1958; King & Meyer, 1958). This hyper-emotionality is manifested by violent attack or flight reactions in response to previously neutral or innocuous stimuli. There is extreme muscular tension to a degree rarely, if ever, noted in the normal animal. Therefore, it is possible that the septal nuclei exert an important function in the "physiological tranquillization" of rats. Destruction of the septal nuclei results in the excited animal because the septum normally counterbalances a stimulatory area.

Chlorpromazine has been shown to cause an activity shift in the opposite direction to that produced by septal destruction (Hunt, 1957). This author showed that, following chlorpromazine administration, both septal operated and unoperated rats were depressed.

A study of the activity of chlorpromazine in rats with surgically destroyed septal nuclei has been made with a view to testing the hypothesis that the septal area "counterbalances" a stimulatory area of the brain. To locate the site of actions of chlorpromazine the extent of the effect of the drug on animals which were septally hyperactive was observed, and compared with the effect of chlorpromazine on normal rats and on control animals which had had a "sham" operation.

METHODS

Animals. Lashley strain hooded rats were bred with Sprague-Dawley albino rats, and the second generation of this hybrid used. Preliminary investigations showed that these hybrid animals had greater physical endurance, being able to swim twice as long as their albino forebears. They were more resistant than the albinos to post-operative infection, to barbiturate sedation and were more docile than the Lashley strain. Animals with albino physical characteristics were discarded and only those animals with the typical hooded features were used.

Thirty-six young adult rats (90 to 120 days old) of each sex were divided into three groups composed of (*a*) 24 which had septal destruction, (*b*) 24 unoperated controls and (*c*) 24 receiving a sham operation.

The three groups of animals were rated as to their activity, and were then given 8 mg/kg of chlorpromazine hydrochloride intraperitoneally on each of the test days. The activity rating of each of the animals was again determined 30 min later. Each control rat was tested for activity rating for a total of 10 days. Those undergoing surgery were given 3 days of pre-operative and 7 days of post-operative testing.

Previous investigations here had shown that the amount of chlorpromazine used (8 mg/kg) and the time between administration and rating (30 min) were optimal.

Activity ratings. An activity rating scale of six components was used to evaluate the behaviour of each animal. It had been adapted with certain minor modifications from one designed by Brady & Nauta (1953), and later modified by King (1958). The term "activity rating" has been substituted for "emotionality rating," for the present authors believed that activity was being measured as a direct result of emotionality, rather than emotionality itself.

The six components of the rating scale were (*a*) reaction to object presentation (a probe presented close to the snout), (*b*) response to a light tap on the back with the probe, (*c*) resistance to capture (slowly approaching the animal and grasping it firmly but not roughly), (*d*) resistance to handling, including a gross evaluation of muscle tone, (*e*) vocalization during the stages of capture and handling, and (*f*) urination and defaecation in response to capture and handling.

Each of the preceding components of the scale was evaluated on a six-point scale with scores from 0 to 5. An overall score was assigned to each animal by totalling the six scores. Each animal had the potential of attaining any score between 0 and 30. The activity ratings reported are average values from each group of 24 rats.

The result of trial runs made to train the observer were in accord with those previously obtained by King (1958) and King & Meyer (1958).

Surgical procedure. Veterinary pentobarbitone sodium (1 part) and 10% ethyl alcohol (8.5 parts) were mixed and general anaesthesia induced by injecting intraperitoneally 0.01 ml. of the mixture for each 1 g of body weight. Atropine sulphate (1 mg/100 g) was administered intraperitoneally to prevent excessive secretion.

After incising the skin of the dorsal surface of the head, the tissue overlying the skull was gently removed. A single trephine hole was drilled over the points at which the lesion was to be made. The co-ordinates used were 2.5 mm anterior to the bregma, 0.5 mm bilateral from the midline, and 6.38 mm beneath the surface of the cerebral hemispheres. Bilateral electrolytic lesions of the septal region were placed by means of a stereotaxic instrument (Baltimore Instrument Co.). A stainless steel electrode insulated except for 0.75 mm at the tip was passed into the brain, and a direct current of 2 mA passed for 20 sec. The circuit was completed by rectal cathode. The wound was then closed by suture with surgical silk, and 30,000 u. of procaine penicillin administered intramuscularly.

The "sham" operation was identical in every respect to the actual surgery performed, but no current was passed through the electrode inserted into the brain after the "sham" operation.

Histological procedure. At the conclusion of the testing period, the entire brains of one-third of the rats selected at random from each group were fixed in formalin initially by injecting a 10% solution intracardially, and then immersing the brains in 37% formalin for at least 24 hr. The brains were then removed from formalin, washed in running water for 24 hr, and dehydrated with successively increasing concentrations of alcohol. The brains were then cleared with xylene, embedded, mounted, sectioned at 10 μ, and stained with cresylecht violet or thionin. Every tenth section was placed on a slide and the focus and extent of the electrolytic lesion estimated.

Statistical analysis. The results obtained were analysed statistically using a design designated as a Type I " Mixed " Design (Lindquist, 1953). Since the F-ratios for variance between groups, within groups, and interaction were significant, the t test for " critical difference," d (Lindquist, 1953), was applied for indication of differences between means.

RESULTS

Activity ratings. The normal activity rating for these rats was 7.89 (7.36 to 8.71). After chlorpromazine (8 mg/kg) the activity rating of normal animals decreased to 2.99 (2.21 to 3.86). In animals with the septum destroyed, the activity rating rose sharply to 20 (15 to 28) on post-operative day 1. This extreme activity gradually decreased until, on post-operative day 7, and for approximately six weeks thereafter, the activity rating was 9.01 (8.24 to 10.86), slightly but significantly above the normal value of 7.89. The mean septal activity for the seven-day post-operative testing period was 14.68 (10.86 to 16.11). On the first post-operative and on all subsequent days of rating, the activity of the septal animals was reduced to 3.03 (1.57 to 4.00) by chlorpromazine.

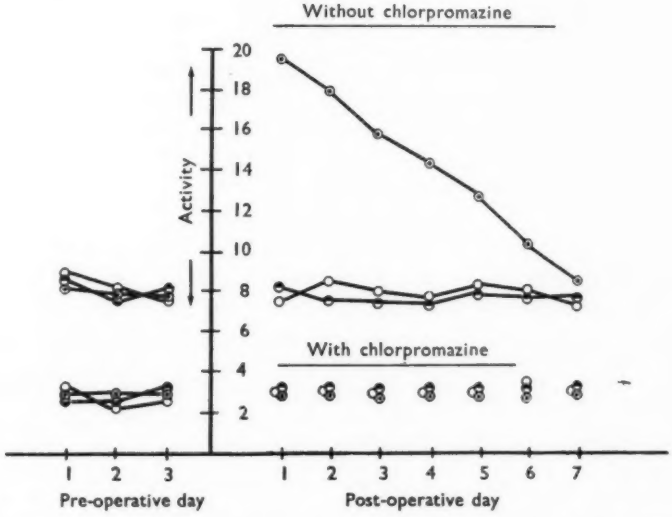

Fig. 1. Mean daily activity of rats before and after septal operation. Septally-operated rats ○——○; sham-operated rats ●——●; and normal rats ○——○. Ordinate: activity in arbitrary units. Abscissa: time in days before and after the operation. Upper three curves refer to untreated animals; lower three curves to chlorpromazine treated animals.

The "sham" operated rats, normal rating 7.93 (7.14 to 9.00), when treated with chlorpromazine, whether pre-operatively or post-operatively, had their activity ratings reduced to 3.05 (2.58 to 3.57). It was striking that the activity ratings of all animals, whether operated or not, were reduced to a value of about 3 by the administration of 8 mg/kg of chlorpromazine. These results are summarized in Fig. 1.

Statistical evaluation. The results were examined statistically for significant differences beyond the 0.01 level of confidence. There was a significant difference between the septally-operated group, the control operated and normal groups before treatment with chlorpromazine ; there was no significant difference between operated control and normal groups, and no significant difference between any of the groups after being treated with chlorpromazine.

TABLE 1

SUMMARY OF A LINDQUIST TYPE I "MIXED" DESIGN ANALYSIS OF VARIANCE OF THE ACTIVITY OF NORMAL, OPERATED CONTROL AND SEPTAL HYPERACTIVE ANIMALS

* Significant at the 1% level of confidence

Source	df	Sums of squares	Mean square (variance)
Between rats	71	368·79	
B	2	367·29	183·65*
Error (b)	69	1·50	0·02
Within rats	72	2,292·72	
A	1	1,835·77	1,835·77*
AB	2	366·18	183·09*
Error (w)	69	90·67	1·31
Total	143	2,661·51	

As the significant F-ratio demonstrated significant difference between groups, within groups, and interaction (Table 1), the *t* test for "critical difference" d was applied for indication of difference among means. The formula by which d is calculated is:

$$d = t\sqrt{\frac{2MS\,(v)}{n}}$$

where the "critical difference" is determined by multiplying *t* at a certain level of confidence and degrees of freedom by the square root of twice the mean square variance, divided by n, the number of subjects.

Histological studies. Microscopic examination of the brains of rats in the group in which the septum had been destroyed surgically showed extensive damage to the septal region. Massive damage was sustained by the precommissural portion of the septum, and heavy damage sustained by the supracommissural portion. In many instances the post-commissural portion was undamaged, leaving intact the post-commissural fornix system. The anterior commissure was always undamaged.

DISCUSSION

Our results confirm the findings of Brady & Nauta (1953), King (1958) and King & Meyer (1958) that septal destruction causes an increase in affective behaviour

and emotionality, and are in agreement with the conclusions of Hunt (1957) that chlorpromazine depresses the activity of normal rats and those with damage in the septal region.

If it is assumed that the septal area functions as a " quiescent " area or is related to the function of calming, it is of interest to inquire into the basic anatomical design of the septal area and to seek the system with which it is in functional balance.

The septal area is thought to have as its function, or one of its functions, a type of depression usually termed emotional depression. With destruction of the septal nuclei, there is an immediate and violent increase in activity, possibly due to a mechanism in the rat which parallels emotionality in the human. With the passage of time, a release phenomenon seems to occur, and the animal gradually returns to a near-normal state. Our results do not support the view that there is any localization of depressive function within the septal nuclei even though the nuclei exist as a discrete, accurately localized histological entity.

The rats used did not return to a completely normal state of activity during the period of observation up to six weeks, so it is highly probable that the septal nuclei do play some, but not a very important, role in " emotional quiescence." Our results suggest that the septal nuclei are a " bridge " over which emotional impulses normally pass. With destruction of this " bridge," the impulses are gradually re-routed until a near normal state is achieved.

The drastic increase in activity after destruction of the septal nuclei could be due to a functional imbalance in the mechanism of the animal's emotionality. It would seem, then, that there is a discrete area, or groups of areas, which are concerned with the function of stimulation of the animal. In recent years many workers have postulated that the amygdaloid complex of nuclei is most likely to be the area that is in functional balance with the septal nuclei (Brady, Schreiner, Geller & Kling, 1954 ; Karli, 1956 ; Schreiner & Kling, 1953 ; Woods, 1956 ; King & Meyer, 1958). This being so it follows that the former area is highly stimulatory in function, and that its ablation would cause profound depression, an effect found by several authors (Brady *et al.*, 1954 ; Karli, 1956 ; Schreiner & Kling, 1953 ; Woods, 1956). Depression of the amygdaloid by chlorpromazine should then be an effective means to combat septal hyperactivity and could explain the lack of significant difference among all the groups treated with chlorpromazine.

By depressing the amygdaloid complex to a constant degree in all animals, a certain percentage of its stimulatory function would be disrupted. In all animals, this disturbance of function would be constant. In the normal and operated control animals, the depressant portion of the emotional cycle is functioning normally, so that the animals exhibit a rating level of about 8. The balance is absent in those whose septa are destroyed with the resulting rating, 1 day post-operative, of about 20. Both levels of emotional activity are presumably being caused by constant stimulation from the amygdaloid complex. The explanation for septal hyperactivity is simply a lack of functional balance. With depression of the amygdaloid complex in all animals, to a constant degree, emotional activity will drop to a constant level, for stimulatory activity is being equally suppressed in all animals.

This work also suggests that there is a possibility of a base line to which emotional activity may be lowered, and it is of interest to speculate whether the dose of 8 mg/kg of chlorpromazine intraperitoneally giving an activity rating of about 3 brought the rats to this base line of activity. This dose has been shown by Rothwacks (1958) to be the most active dose of chlorpromazine within the range from 2 mg/kg to 11 mg/kg. For this reason the present authors assumed that 8 mg/kg was the maximal effective dose, and would presumably depress the activity of the amygdaloid complex maximally.

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EFFECT OF THYROID HORMONES ON HISTAMINE FORMATION IN THE RAT

BY

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Treatment with thyroxine or liothyronine increased the urinary excretion of free histamine in male and female rats under the influence of aminoguanidine, a histaminase inhibitor. There was no evidence for an altered catabolism of histamine in rats treated with liothyronine, since the same percentage of subcutaneously injected [^{14}C]-histamine appeared unchanged in the urine. After subcutaneous injection of [^{14}C]-L-histidine, rats treated with liothyronine excreted more [^{14}C]-histamine than control rats. An increased formation of [^{14}C]-histamine from [^{14}C]-histidine could also be demonstrated *in vitro*, in the glandular portion of the stomach, while no increase was found in the skin.

The metabolism and actions of histamine may be influenced by a number of endocrine glands. The relationship between histamine and the adrenocortical hormones has been most studied (Rose & Browne, 1941 ; Haeger & Kahlson, 1952 : Schayer, 1956 ; Telford & West, 1960), but there are also suggestions of an effect of thyroid hormones on histamine metabolism. Thus Gotzl & Dragstedt (1940) and Feldberg & Loeser (1954) found the histamine content of the skin to be high in hyperthyroid states in rat and in man, whereas the skin histamine was low in myxoedema. Parratt & West (1960) found an increase in the urinary excretion of histamine in the rat after treatment with L-thyroxine, and attributed this to a decrease in histaminase activity. They found no increase in the tissue histamine in rats treated with thyroxine.

It is known that there may be a connexion between adrenocortical and thyroid function. During studies on the effect of adrenalectomy on urinary histamine in the rat (Angervall, Bjurö & Westling, 1961) the question arose whether changes in thyroid activity could be responsible for the effects observed. The action of L-thyroxine on the urinary excretion of histamine in rats treated with the histaminase inhibitor aminoguanidine was therefore examined. If the increase in urinary histamine reported by Parratt & West (1960) was entirely due to diminished histaminase activity, it should not occur in rats treated with aminoguanidine.

METHODS

White rats of the Sprague-Dawley strain were given a constant amount (10 g daily) of a semi-synthetic food essentially free from histamine (Kahlson, Rosengren & Westling, 1958). For urine collection the animals were kept in metabolism cages, and the urine collected

under hydrochloric acid in 24-hr portions. Aminoguanidine sulphate was given subcutaneously once daily in a dose of about 20 mg/kg. The excretion of free histamine was determined by bioassay on the guinea-pig ileum as described (Angervall, Bjurö & Westling, 1961). Values are given as μg of free histamine base excreted per 24 hr.

Histamine content of skin was estimated by bioassay after extraction according to Code (1937).

[^{14}C]-Histamine was injected subcutaneously in a dose of about 10 μg. The [^{14}C]-histamine content of the urine was measured with the isotope dilution technique described by Lindell & Schayer (1958). 1 ml. of the 24-hr sample of urine was taken and 40 mg histamine base added as carrier. The histamine was then extracted with alkaline butanol and crystallized as the dipicrate. After removal of the picric acid, histamine was made to react with *p*-iodophenylsulphonyl chloride (pipsyl chloride). The radioactivity of the pipsylhistamine formed was measured on standard plates at infinite thickness under standardized conditions in a flow counter. The pipsylhistamine was recrystallized until the radioactivity was constant.

Histamine formation *in vivo* was determined by analysis of the urinary excretion of [^{14}C]-histamine during 24 hours after the subcutaneous injection of [^{14}C]-L-histidine. The urine specimen was divided in two halves, and histamine-histidine carrier (40 mg histamine base and 50 mg L-histidine monohydrochloride as diluent) was added to each half. Histamine (plus the small amount of [^{14}C]-histamine) was then extracted with butanol, crystallized as the dipicrate and then converted to pipsylhistamine and recrystallized until its radioactivity was constant.

Histidine decarboxylase activity of abdominal skin and the glandular portion of the stomach was determined *in vitro* with a modification of Schayer's technique (Kahlson, Rosengren, Westling & White, 1958). The amount of [^{14}C]-histamine formed from [^{14}C]-histidine by minced tissue was determined after purification and crystallization as histamine dipicrate and pipsylhistamine. The incubation was carried out under nitrogen for 3 hr at 37° C. in a shaking incubator. Skin (1.0 g minced tissue) was incubated with 1.5 ml. 0.1 M sodium phosphate buffer of *p*H 7.4, 12.4 μg [^{14}C]-L-histidine and 0.1 ml. 10^{-3} M aminoguanidine (to inhibit histaminase), and the glandular portion of the stomach (0.7 to 0.9 g minced tissue) with 1.0 ml. buffer, 12.4 μg [^{14}C]-L-histidine and 0.1 ml. 10^{-3} M aminoguanidine. The entire stomach wall of the glandular portion was used for incubation. Blanks were obtained by adding semicarbazide (a powerful histidine decarboxylase inhibitor), to give a final concentration of about 2×10^{-2} M. After incubation for 3 hr, histamine-histidine carrier (40 mg of histamine base and 50 mg. of L-histidine monohydrochloride as diluent) was added, and allowed to mix well. The proteins were precipitated with trichloroacetic acid. Histamine was then extracted and converted to pipsylhistamine as described by Kahlson *et al.* (1958b). Pipsylhistamine was recrystallized until its radioactivity was constant. Histidine decarboxylase activity is given as counts/min of [^{14}C]-histamine formed by a certain amount of tissue in 3 hr, with blank values and background radioactivity subtracted.

[^{14}C]-Radioactivity was measured at infinite thickness under standardized conditions in a flow counter with a background of 21 to 23 counts/min.

The radioactive substances were obtained from the Radiochemical Centre, Amersham, England: [^{14}C]-histamine, labelled in the 2 position of the imidazole ring, specific activity 39.4 μc/mg; 1 μg giving about 2,500 counts/min with 40 mg histamine base as carrier and measured as pipsylhistamine under our conditions for measurements of radioactivity; [^{14}C]-L-histidine, labelled at the same carbon atom as the radioactive histamine, specific activity 63.0 μC/mg; 1 μg of [^{14}C]-histamine formed from this [^{14}C]-histidine would give about 5,500 counts/min with 40 mg. histamine base as carrier.

Aliquots of the solutions of [^{14}C]-histamine and [^{14}C]-histidine used for injection were extracted for their content of [^{14}C]-histamine with the same methods as used for the urine samples.

L-Thyroxine and liothyronine (L-3,5,3-triiodothyronine) were used as the sodium salts, manufactured by Nyegaard, Oslo, and kindly supplied by Erco, Stockholm, Sweden. As recommended by the manufacturers, the hormones were first dissolved in 0.1 N sodium

hydroxide and 0.1 N hydrochloric acid then added to give a *p*H of about 9.7. The daily dose of hormones was injected in a volume of 0.2 to 0.6 ml. Control rats were given the same volume of solvent (sodium hydroxide with hydrochloric acid). This is referred to as sodium hydroxide.

Mean values are given together with the standard error of the mean (mean ± s.e.m.).

RESULTS

1. *Urinary excretion of free histamine*

The urinary excretion of free histamine in response to thyroxine and liothyronine was examined in 7 rats, which were also given aminoguanidine. It was found that both hormones caused a conspicuous increase in the urinary histamine, which started some 3 to 5 days after the beginning of injections, and continued for 7 to 10 days after the discontinuation of treatment (Fig. 1). A similar effect was not obtained with sodium hydroxide (Figs. 1 and 2). The experiments were not designed to compare the effectiveness of the two thyroid hormones but liothyronine seems to be rather more effective (Table 1). With both hormones, in various dosages, there appeared to be a parallelism between the effect on the body weight and the effect on the urinary histamine.

The effect of thyroid hormones was seen both in male and female rats (Figs. 1 and 2, Table 1). Due to the lower initial level of urinary histamine in the males, the increase brought about by the thyroid compounds appeared to be more pronounced in this sex, but the absolute increase was about the same, or slightly larger, in the females.

Fig. 2 shows that the effect of the thyroid hormones on the urinary histamine does not diminish on continued treatment. In the rat shown in Fig. 2 about 200 μg excess histamine per day appeared in the urine even after 26 days of treatment with thyroxine.

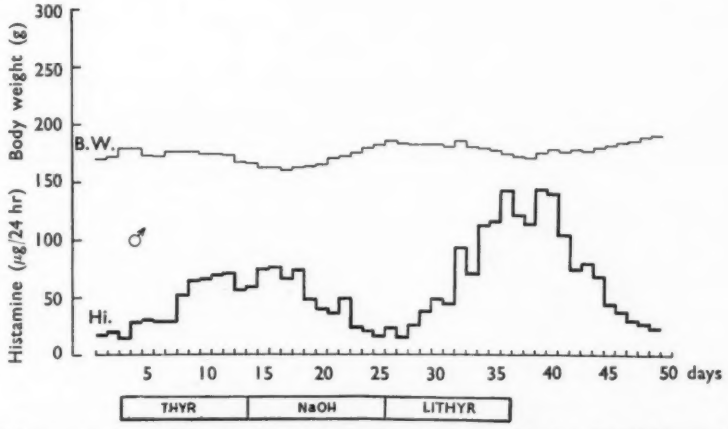

Fig. 1. Urinary excretion of histamine in μg/24 hr (Hi.) and body weight in g (B.W.) in a male rat during treatment with 0·2 mg thyroxine (THYR), 0·2 ml. sodium hydroxide (NaOH) or 0·04 mg liothyronine (LITHYR), subcutaneously, once daily. The rat received in addition aminoguanidine 20 mg/kg subcutaneously once daily.

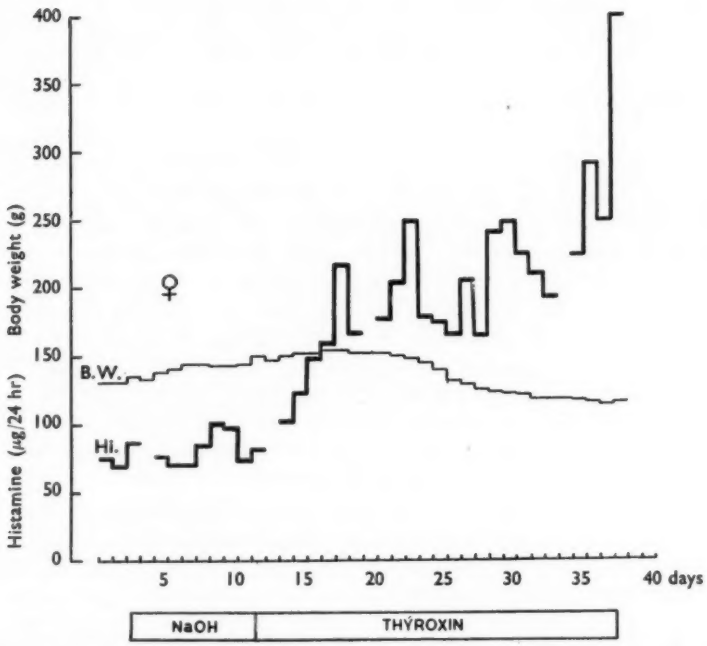

Fig. 2. Urinary excretion of histamine in μg/24 hr (Hi.) and body weight in g (B.W.) in a female rat during treatment with 0·2 ml. sodium hydroxide (NaOH) or 0·6 mg thyroxine (THYROXIN), subcutaneously once daily. The rat received in addition aminoguanidine 20 mg/kg subcutaneously once daily

TABLE 1

EFFECT OF THYROID HORMONES ON URINARY HISTAMINE

Mean values are given for the urinary excretion of histamine during three days before treatment, and during the 7th, 8th and 9th day of treatment, and expressed in μg/24 hr. The animals were injected subcutaneously once daily with thyroxine, 0·2 or 0·6 mg, liothyronine, 0·04 or 0·12 mg, or with sodium hydroxide of the same *p*H and volume as the hormone solutions. All rats received in addition aminoguanidine, 20 mg/kg subcutaneously once daily

			Urinary non-isotopic histamine (μg/24 hr)				
			During treatment with				
				Thyroxine		Liothyronine	
Rat no.	Sex	Before treatment	NaOH 0·2 ml.	0·2 mg	0·6 mg	0·04 mg	0·12 mg
1	Female	91	—	139	—	—	323
2	Female	99	—	—	164	—	311
3	Female	77	84	—	158	—	—
4	Male	18	—	69	—	99	—
5	Male	12	14	—	84	—	161
6	Male	19	—	75	—	88	—
7	Male	11	9	—	76	—	—

Thyroxine and liothyronine thus caused a pronounced and sustained increase in the urinary histamine in rats treated with aminoguanidine.

2. *Catabolism of injected [^{14}C]-histamine*

The effect of liothyronine on the catabolism of histamine was examined in 1 female and 3 male rats, which were compared with litter-mate controls. The results are given in Table 2. The excreted [^{14}C]-histamine in % of the injected [^{14}C]-histamine was the same in the liothyronine group as in the control group.

TABLE 2

EFFECT OF LIOTHYRONINE ON CATABOLISM OF INJECTED [^{14}C]-HISTAMINE

8 litter-mate rats were injected once daily for 8 days with 0·12 mg liothyronine or with sodium hydroxide of the same *p*H and volume as the hormone solution. All rats received in addition aminoguanidine, 20 mg/kg subcutaneously on the 7th and 8th day. At the 8th day all rats were given 10 μg of [^{14}C]-histamine subcutaneously and urine collected for 24 hr. Values are given as excreted [^{14}C]-histamine in % of injected [^{14}C]-histamine. Values for urinary excretion of non-isotopic histamine are given as μg/24 hr

Rat no.	Sex	Treatment	Body weight		[^{14}C]-Histamine in urine	Urinary non-isotopic histamine μg/24 hr
			Initial	Final		
1	Female	Liothyronine	186	169	35	348
2	Male		180	147	18	133
3	Male		176	144	13	218
4	Male		152	131	24	204
5	Female	NaOH	167	176	37	156
6	Male		166	179	21	39
7	Male		176	186	20	43
8	Male		186	190	16	42

The female rats excreted about twice as much of the injected [^{14}C]-histamine in unchanged form as the male rats. The values observed are of the same magnitude as found in normal rats by Westling & Wetterqvist (unpublished results).

It may be seen from Table 2 that the treatment with liothyronine had caused about the same increase in the urinary excretion of non-isotopic histamine as recorded previously (section 1). Still, the catabolism of [^{14}C]-labelled histamine, as reflected by its urinary excretion, was not measurably changed.

3. *Urinary excretion of [^{14}C]-histamine after subcutaneous injection of [^{14}C]-histidine*

Information about the rate of histamine formation from histidine in the intact rat can be obtained by analysis of the urinary [^{14}C]-histamine after subcutaneous injection of [^{14}C]-L-histidine. Such experiments were performed on 4 female litter-mate rats, and the results are shown in Table 3.

It may be seen that the 2 rats treated with liothyronine had a substantially larger excretion of free, non-labelled histamine, as shown previously in section 1. In addition, their excretion of [^{14}C]-histamine after injection of [^{14}C]-histidine was about twice as large as that of the control rats. There was thus some parallelism between the excretion of non-labelled histamine and of [^{14}C]-histamine.

TABLE 3

URINARY EXCRETION OF [^{14}C]-HISTAMINE AFTER SUBCUTANEOUS INJECTION OF [^{14}C]-HISTIDINE

4 litter-mate rats were injected once daily for 8 days with 0·12 mg liothyronine or with sodium hydroxide of the same *p*H and volume as the hormone solution. At the 8th day aminoguanidine (20 mg/kg) and 30 min later about 450 μg of [^{14}C]-histidine were injected subcutaneously and the urine collected for 24 hr

Rat no.	Treatment	Body weight		Urinary excretion of [^{14}C]-histamine counts/min/24 hr		Urinary non-isotopic histamine μg/24 hr
		Initial	Final	Duplicate assays	Average	
14	Liothyronine	138	132	1,540 1,620	1,580	258
15		134	132	1,640 1,730	1,690	218
16	NaOH	134	159	600 610	605	89
17		131	148	900 820	860	132

Unfortunately the [^{14}C]-histidine available to us contained a small amount of [^{14}C]-histamine, as judged by analysis of the original solution used for injection into the rats (compare White, 1960). The amount of "preformed" [^{14}C]-histamine thus injected was calculated to correspond to 360 counts/min. It is known that in female rats given aminoguanidine about 1/3 of subcutaneously injected [^{14}C]-histamine is excreted unchanged into the urine (section 2 above; Westling, 1958; Westling & Wetterqvist, unpublished). From the total [^{14}C]-histamine in the urine 120 counts/min should therefore be subtracted in order to obtain a closer estimate of the amount actually formed from [^{14}C]-histidine by the rat.

The increase in urinary excretion of non-isotopic histamine during treatment with liothyronine was thus accompanied by an increased excretion of [^{14}C]-histamine after subcutaneous injection of [^{14}C]-histidine. This could be due to a more rapid formation of [^{14}C]-histamine, or to a deficient catabolism of [^{14}C]-histamine, formed at a normal rate. The demonstration of an unchanged catabolism of injected [^{14}C]-histamine during treatment with liothyronine (section 2) speaks strongly in favour of the former possibility.

4. *Histidine decarboxylase activity in the skin and stomach*

The results so far obtained indicated that the rats treated with thyroid hormones formed histamine from histidine at a higher rate than normally. To see where this increased formation of histamine took place, *in vitro* experiments were performed. The histamine formation in abdominal skin and stomach was examined, since Schayer (1956) has found that adrenalectomy and administration of adrenocortical steroids elicit changes in the histamine-forming capacity of these tissues, the stomach reacting in a reverse manner to the skin.

The effect of liothyronine on histamine formation *in vitro* was examined in 2 groups of 11 female rats, the urine of which was not collected. The results are given in Table 4. Liothyronine caused a loss in body weight. The histidine decarboxylase activity of the glandular part of the stomach was about twice as

TABLE 4

EFFECT OF LIOTHYRONINE ON HISTIDINE DECARBOXYLASE ACTIVITY

11 female rats were injected with 0·1 mg liothyronine subcutaneously once daily for 7 days; a control group of 11 female rats received the same volume of sodium hydroxide. Mean values ±s.e.m. are given. * 6 rats. † 5 rats.

Treatment	Body weight g		Histidine decarboxylase activity			Histamine content of abdominal skin μg/g
	Initial	Final	Glandular stomach counts/min	Glandular stomach counts/min/g tissue	Skin counts/min/g tissue	
Group A: controls 0·2 ml. sodium hydroxide for 7 days. 11 rats	153	175	1,390±278	1,890±395	21·5±1·81	25·3±2·92*
Group B: liothyronine 0·1 mg for 7 days. 11 rats	160	153	2,690±272	3,840±343	20·2±2·09	35·0±1·89†

large in the liothyronine-treated group, and the difference between this group and the control group (given sodium hydroxide) was statistically significant with $P<0.01$. The mean weight of the glandular stomach was slightly lower in the liothyronine group (0.694 ± 0.022 g) than in the control group (0.752 ± 0.022 g). The difference in histidine decarboxylase between liothyronine-treated and control rats was therefore even larger when the activity was calculated per g of tissue.

There was no difference between the histidine decarboxylase activity in the skin from liothyronine-treated rats and control rats (Table 4), but the actual content of non-isotopic histamine appeared to be larger in the liothyronine group.

DISCUSSION

The finding of an increased urinary excretion of free histamine during treatment with thyroid hormones confirms the observation by Parratt & West (1960). However, the increase in urinary histamine is not likely to be caused by a diminished histaminase activity, since it occurred also in the present experiments, in which the action of histaminase (diamine oxidase) was inhibited by the administration of aminoguanidine during the whole experimental period. Aminoguanidine, in the dose given here, inhibits histaminase relatively completely (Schayer, Wu & Smiley, 1954; Westling, 1958).

The thyroid hormones could, however, also interfere with another enzymatic pathway for the inactivation of histamine, namely, the enzyme responsible for the methylation of histamine. This enzyme is active also in rats given aminoguanidine (for example, Westling, 1958). However, there was no evidence for diminished methylation of histamine in response to liothyronine, since there was no change in % of injected [^{14}C]-histamine excreted in the urine in an unchanged form.

As causes of the elevation of urinary histamine during treatment with thyroid hormones, we may also consider: (1) increased intake of histamine, (2) release of histamine normally present in the body, and (3) increased formation of histamine. Of these (1) and (2) can be excluded with considerable certainty, the former because the animals were given a rigidly fixed intake of food with a constant composition.

If the thyroid hormones released histamine from the tissues one should expect a lowering of the tissue histamine content. The available observations indicate that tissue histamine is, if anything, higher than normal in " hyperthyroid states " (Gotzl & Dragstedt, 1940 ; Feldberg & Loeser, 1954 ; Parratt & West, 1960). Furthermore, a mere release of histamine should be transient if unaccompanied by an increased formation of the amine. The sustained increase in urinary histamine during prolonged treatment with thyroid hormones is apparent from Fig. 2 in the present paper.

By exclusion, increased formation with subsequent excretion is thus the most likely explanation for the high urinary output of histamine in rats treated with thyroid hormones. Direct support for this hypothesis is provided by the experiments with [^{14}C]-histidine. The finding of increased amounts of [^{14}C]-histamine in the urine after injection of [^{14}C]-histidine does not by itself prove that the rate of histamine formation is increased during treatment with liothyronine, but, taken together with the finding of an unchanged catabolism of [^{14}C]-histamine, it would seem to provide very strong evidence for a more rapid formation and turnover rate in the rat as a whole.

Supplementary evidence for increased histamine formation under the influence of liothyronine was found in the glandular part of the stomach ; this tissue formed histamine *in vitro* at about twice the rate of the controls. In the skin, however, there was no measurable increase of the histamine formation *in vitro*. This is somewhat surprising, since both the present experiments and those of Gotzl & Dragstedt (1940) and Feldberg & Loeser (1954) indicate that the skin contains more histamine in " hyperthyroid " states. However, Parratt & West (1960) found no change in skin histamine in rats treated with thyroxine.

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ACTION OF CHLOROTHIAZIDE ON THE DISTRIBUTION, EXCRETION AND HYPOTENSIVE EFFECT OF PEMPIDINE IN MAN

BY

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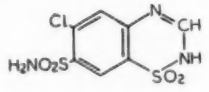
When chlorothiazide is given to hypertensive patients who are receiving pempidine a rise in plasma pempidine concentration occurs and this is proportionately greater than the additional fall in blood pressure. After pempidine has been added to human whole blood *in vitro* or *in vivo* the ratio of the pempidine concentration in the red cells to that in the plasma falls in the course of 1 hr from an initial value greater than 2 to about 1.2. If chlorothiazide is present also, however, the ratio remains constant at 0.7. Changes in the plasma pempidine concentration *in vivo* probably result from the binding of pempidine to plasma protein in the presence of chlorothiazide. This has been observed *in vitro* by a dialysis technique.

The principal action of chlorothiazide (6-chloro-7-sulphamoyl-1,2,4-benzothiadiazine 1,1-dioxide, I) is to increase the excretion of sodium, chloride and water. It also enhances the response obtained from a number of drugs used to lower blood pressure and is consequently of value in the treatment of hypertension (Freis & Wilson, 1957; Hollander & Wilkins, 1957). Several mechanisms have been proposed for this second effect although it is now generally considered to result from the reduction of plasma volume following sodium diuresis (Dustan, Cumming, Corcoran & Page, 1959; Dollery, Harington & Kaufmann, 1959; Wilson & Freis, 1960). It is possible, however, that chlorothiazide also affects the excretion of hypotensive drugs because it raises the urinary *p*H, by inhibiting carbonic anhydrase, and may therefore influence excretion by non-ionic diffusion in the renal tubules (Harington & Kincaid-Smith, 1958).



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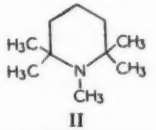

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Pempidine (1,2,2,6,6-pentamethylpiperidine, II) is a ganglion-blocking agent used in the treatment of hypertension. Recently, during studies of hypertensive patients who were receiving the drug orally, we found that (i) the hypotension obtained paralleled the plasma pempidine concentration and (ii) chlorothiazide caused only a slight increase in the response to a dose of pempidine although it

almost trebled the plasma pempidine concentration (Dollery, Emslie-Smith & Muggleton, 1960). This paper describes further work that we have carried out to elucidate the mechanism of the action of chlorothiazide on the distribution, excretion and hypotensive effect of pempidine in man.

METHODS

Plasma pempidine concentrations in hypertensive patients. The effect of chorothiazide on plasma pempidine concentrations was investigated in patients with severe hypertension. Many had retinal haemorrhages and exudates but none had a blood urea level of more than 50 mg/100 ml. and renal function was not seriously impaired.

Patients given oral pempidine and oral chlorothiazide. Five patients were studied before and after chlorothiazide was added to their daily maintenance treatment with pempidine. Both drugs were given orally and each patient was on a stable regime for at least one week before any observations were made. Pempidine (10 to 100 mg daily) was administered as the hydrogen tartrate in four equal doses at 6 a.m., noon, 6 p.m. and 11 p.m.; chlorothiazide (250 to 2,000 mg daily) was given in two equal doses at 6 a.m. and 6 p.m. Blood pressures (lying and standing) were recorded and blood samples were taken just before the mid-day dose of pempidine and again at 1, 2, 4 and 6 p.m. A sixth patient who had received pempidine and chlorothiazide daily for two months was studied before, and again two weeks after, chlorothiazide was withdrawn.

Patients given intravenous pempidine and oral chlorothiazide. Plasma pempidine concentrations and blood pressures were measured in ten patients for 1 hr following an intravenous dose of pempidine. Each patient lay flat in bed and the blood pressure was recorded at intervals until it became steady. After a control sample of blood had been taken the pempidine (usually 2.5 mg) was given intravenously. Further blood samples were taken and the blood pressure measured 5 (or 10), 30 and 60 min after the injection. From a few patients it was possible to obtain extra samples during the first 5 min. Six patients were given a second injection of pempidine on the following day, after having two oral doses of chlorothiazide (500 mg) in the preceding 15 hr. Three days later three of the patients were again given 2.5 mg pempidine intravenously, but no chlorothiazide. Two patients were first of all given 10 mg pempidine intravenously without chlorothiazide and on a subsequent occasion 2.5 mg after premedication with chlorothiazide.

Patients given intravenous pempidine and diuretics other than chlorothiazide. The effect of other diuretics on pempidine was investigated with the same schedule of blood samples and pressure readings as described above for patients given intravenous pempidine and oral chlorothiazide. Three patients were given mersalyl intramuscularly 8 and 3 hr before the test dose of pempidine. A fourth patient had acetazoleamide orally 8 and 2 hr before the pempidine.

Patients given oral pempidine and intravenous chlorothiazide. Nine patients on maintenance treatment with oral pempidine were each given a single intravenous injection of chlorothiazide (100 to 500 mg as sodium salt) 2 to 6 hr after a dose of pempidine. Blood pressures (lying and standing) were recorded and blood samples taken 1 to 2 hr after the injection of chlorothiazide.

Patients given intravenous pempidine and intravenous chlorothiazide. Pempidine and chlorothiazide (as sodium salt) were mixed in a syringe and administered intravenously to two patients. Two patients had intravenous pempidine 1 hr after intravenous chlorothiazide and in a third the drugs were injected simultaneously but separately. In every case blood pressures and blood samples were taken immediately before and 10, 30 and 60 min after injecting the pempidine.

Renal excretion of pempidine. The renal clearance of pempidine was measured in patients during continuous infusion of about 1 mg of pempidine each hour. Blood samples were taken and the entire urine output was collected during periods of 1 hr before and after chlorothiazide

had been added to the infusion at rates varying from 40 to 100 mg/hr. Preliminary experiments showed that an interval of 1 hr between the two collection periods was enough for stable conditions to be re-established after the addition of chlorothiazide. Similar studies were made without chlorothiazide, but sodium bicarbonate (20 to 100 mEq/hr) was then infused to produce a comparable change in the urinary *p*H. The renal clearance of pempidine was also measured over consecutive hourly periods without the addition of either chlorothiazide or sodium bicarbonate. In none of these studies was the bladder catheterized, but the patient's water intake was increased so that relatively large amounts of urine could be obtained and errors of collection thereby minimized.

Distribution of pempidine in the tissues of the rat. The effect of chlorothiazide on the distribution of pempidine in tissues was studied in rats. Five animals, each weighing about 450 g, were deprived of food for 12 hr, and pempidine (1 mg/kg) was then administered in aqueous solution by stomach tube. Thirty minutes later the rats were killed and bled. The various organs and tissues were removed as quickly as possible and weighed. Control experiments without pempidine were performed under identical conditions. Both experiments were also carried out with rats which were given three equal doses of chlorothiazide (5 mg/kg orally) 7, 4 and 1 hr before the pempidine.

Distribution of pempidine between red cells and plasma in vitro. To investigate the distribution of pempidine between red cells and plasma 40 ml. of freshly-drawn human blood was heparinized and kept at 37° C with occasional stirring. Samples were removed 1, 3, 5, 10, 30 and 60 min after the addition of 25 μg of pempidine dissolved in 1 ml. 0.9% sodium chloride solution. Each sample was centrifuged for the same time (1 min) and at the same speed (3,000 rev/min), so that the red cells and the plasma were separated under standard conditions. Similar experiments were made with blood (*a*) which had stood for 1 hr at 37° C; (*b*) to which 1.37 mg of chlorothiazide had been added 1 hr previously; (*c*) after the addition of 0.005 ml. of mersalyl injection *B.P.*; and (*d*) from a patient who had been pretreated with chlorothiazide.

Binding of pempidine to serum albumin in vitro. So far it has not been possible for us to undertake a comprehensive study of the binding of pempidine to proteins, but some exploratory experiments have been carried out using the technique of equilibrium dialysis. Dialysis sacs were prepared from Visking tubing previously boiled for 1 hr in distilled water to remove soluble impurities. Each sac was filled with 5 ml. of solution containing bovine serum albumin (Fraction V) and chlorothiazide, and was placed in a stoppered tube containing 20 ml. of pempidine solution. The system was allowed to equilibrate for 48 hr at 25° C and the concentrations of pempidine and chlorothiazide in the outer compartment were then measured. In some experiments pempidine concentrations inside the sac were also determined, but we could not obtain reliable measurements of chlorothiazide concentration with the protein-containing solution. If pempidine and chlorothiazide were transported across the membrane entirely by diffusion, then their equilibrium concentrations could readily be calculated from the volumes of solution in the outer and inner compartments. On the other hand, binding of either drug to the protein would disturb the equilibrium and the consequent concentration changes could be used to measure the extent of protein-binding. Six series of experiments were performed.

Pempidine (0 to 20 μg/ml.) and chlorothiazide (0 to 50 μg/ml.) were tested separately in the absence of albumin to show that neither pempidine nor chlorothiazide was bound to the dialysis sac. These experiments were repeated with 1% albumin in the dialysis sac to assess the binding of pempidine and chlorothiazide, separately, to serum albumin. Finally, the effect of chlorothiazide added to the albumin (1%) in the sac on pempidine binding was studied. A fixed concentration (50 μg/ml.) of chlorothiazide was tested with varying concentrations (0 to 1 μg/ml.) of pempidine and various concentrations (0 to 50 μg/ml.) of chlorothiazide were tested with a fixed concentration (1 μg/ml.) of pempidine.

Preparation of samples. Samples of blood were heparinized and centrifuged to separate the red cells and plasma. The red cells were lysed by adding an equal volume of distilled water and the debris removed by centrifugation. Urine was centrifuged and, where required,

its *p*H was measured with a glass electrode. Rat tissues were homogenized in distilled water. After material from five rats had been pooled the final volume was adjusted so that 10 ml. of homogenate was equivalent to 1 g of the original tissue.

Estimation of pemipidine. Concentrations of pempidine in plasma, red cells, tissue homogenates and urine were measured by the eosin fluorescence technique. A brief description of this method has already been given (Dollery *et al.*, 1960) and a full account will appear shortly elsewhere (Ivens & Muggleton, to be published).

Estimation of chlorothiazide. Chlorothiazide was estimated by first hydrolysing with sodium hydroxide and then diazotizing and coupling with *N*-(1-naphthyl)ethylenediamine hydrochloride. The intensity of the colour produced was measured at 520 mμ in 1 cm cuvettes (Baer, Leidy, Brooks & Beyer, 1959).

RESULTS

Plasma pempidine concentrations

Oral pempidine and oral chlorothiazide. When doses of chlorothiazide ranging from 250 to 2,000 mg were added to the daily pempidine treatment there was a slightly greater fall in blood pressure than with pempidine alone, as shown in Fig. 1. In every case the plasma pempidine concentration remained at a high level

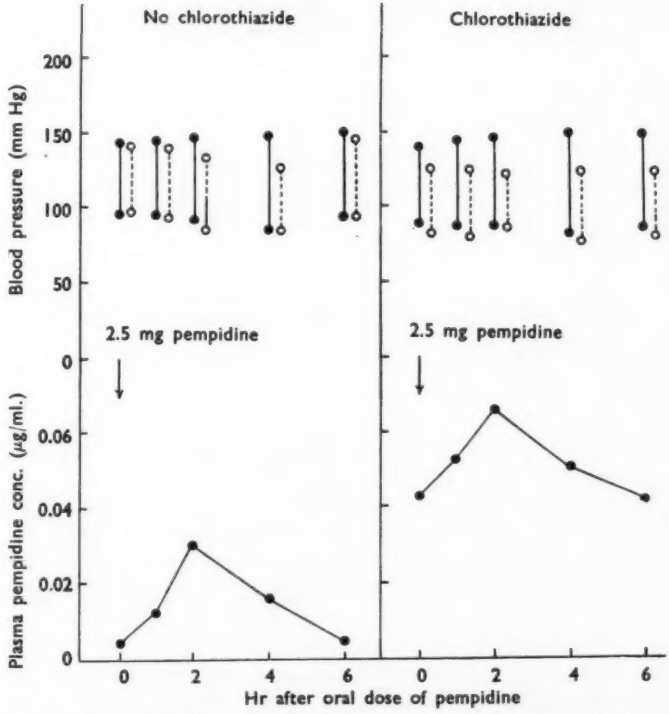

Fig. 1. Effect of chlorothiazide on the plasma pempidine concentration and the hypotensive response after an oral dose of pempidine. Blood pressures (●——● lying and ○- - -○ standing) and plasma pempidine concentrations were measured for 6 hr after one of a series of 6-hourly doses (2.5 mg) of pempidine, with and without 12-hourly doses of chlorothiazide.

throughout the 6 hr period of observation, and the peak value, which occurred about 2 hr after the dose of pempidine, was approximately 2.5 times that found without chlorothiazide. The plasma pempidine concentration in the presence of chlorothiazide was always greater than for the same dose of pempidine given alone. Table 1 contains results for three typical patients. It shows the plasma pempidine concentrations after one of a series of 2.5 mg doses of pempidine, with and without chlorothiazide.

Intravenous pempidine and oral chlorothiazide. After an intravenous injection of pempidine the plasma concentration of the drug fell rapidly during the first 5 min and then more slowly. However, when the patient had previously been given chlorothiazide the plasma pempidine concentration decreased much more slowly and 10 min after the injection it was consistently nearly 7 times greater than that without chlorothiazide. The effect of chlorothiazide on the plasma concentration and hypotensive action of pempidine was very persistent and could be demonstrated 3 days after the last dose of chlorothiazide (Fig. 2).

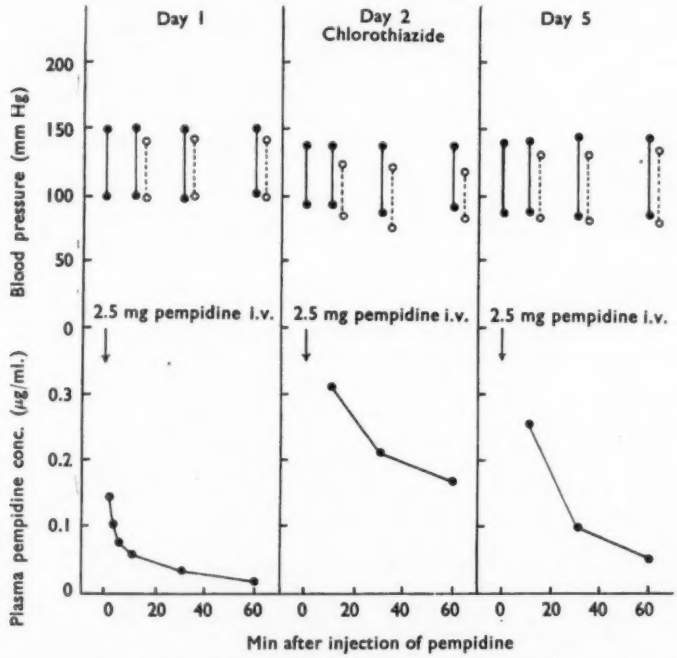

Fig. 2. Effect of chlorothiazide on the plasma pempidine concentration and hypotensive response after an intravenous injection of pempidine. Blood pressures (●——● lying and ○- - -○ standing) and plasma pempidine concentrations were measured for 1 hr after an intravenous injection of pempidine (2.5 mg) on days 1, 2 and 5. The patient received 2 oral doses of chlorothiazide (500 mg) in the 15 hr preceding observations on Day 2. Note elevated plasma pempidine concentrations on Day 2 and Day 5.

TABLE 1

EFFECT OF CHLOROTHIAZIDE ON PLASMA PEMPIDINE CONCENTRATIONS IN HYPERTENSIVE PATIENTS

Plasma pempidine concentrations (μg/ml.) for 6 hr after one of a series of 6-hourly doses (2·5 mg orally), with and without 12-hourly oral doses of chlorothiazide

Time after dose (hr)	Patient 1. Chlorothiazide (mg/day)		Patient 2. Chlorothiazide (mg/day)		Patient 3. Chlorothiazide (mg/day)	
	0	250	0	500	0	1,000
0	0	0·030	0	0·027	0·003	0·040
1	0·008	0·050	0·009	0·036	0·011	0·055
2	0·030	0·069	0·018	0·045	0·030	0·085
4	0·015	0·055	0·008	0·027	0·013	0·065
6	0·002	0·041	0·002	0·030	0·004	0·043

The effect of chlorothiazide on the distribution of pempidine between red cells and plasma *in vivo* is of great interest. Table 2 shows concentrations of pempidine in plasma and red cells and the corresponding values of the distribution ratio observed in a patient over five days.

TABLE 2

EFFECT OF ORAL CHLOROTHIAZIDE ON CONCENTRATIONS IN PLASMA AND RED CELLS OF PEMPIDINE INJECTED INTRAVENOUSLY

Concentrations of pempidine (μg/ml.) in plasma (P) and red cells (R.C.), and their ratio, for 1 hr after intravenous injection of the drug. Day 1=2·5 mg pempidine; Day 2=2·5 mg pempidine after premedication of the patient orally with chlorothiazide; Day 5=2·5 mg pempidine

Time after pempidine injection (min)	Day 1			Day 2			Day 5		
	P	R.C.	R.C./P	P	R.C.	R.C./P	P	R.C.	R.C./P
1	0·326	0·628	2·09	0·384	0·294	0·765	0·340	0·331	0·974
5	0·269	—	—	0·318	0·221	0·695	0·246	0·244	0·991
10	0·059	0·111	1·88	0·258	0·178	0·669	0·202	0·204	1·01
30	0·034	0·065	1·91	0·199	0·138	0·692	0·160	0·152	0·950
60	0·022	0·028	1·27	0·131	0·094	0·717	0·118	0·105	0·890

On Day 1, 2.5 mg of pempidine was given intravenously and blood samples were taken at intervals for 1 hr. The distribution ratio decreased from 2.09 immediately after the injection to 1.27 1 hr later. The patient next received chlorothiazide orally and another 2.5 mg of pempidine was injected on Day 2. The distribution ratio then had a value which was practically constant (about 0.7). No further treatment was given until a third injection (2.5 mg) of pempidine was administered on Day 5, when an almost constant ratio (about 1) was obtained. On Days 2 and 5, therefore, relatively more of the pempidine was in the plasma than on Day 1, and, although the distribution ratio was slowly returning to its normal value, the effect of chlorothiazide could still be detected even 3 days after the last dose. Measurements more than 3 days after cessation of chlorothiazide were not attempted. Note that the distribution ratio in the presence of chlorothiazide was nearly constant, whereas a steadily decreasing ratio was observed in the absence of chlorothiazide.

Two patients were given 10 mg of pempidine intravenously, and on another occasion 2.5 mg after premedication with chlorothiazide. The larger dose of pempidine gave a greater hypotensive response although the plasma pempidine concentration was less than that following the smaller dose administered after chlorothiazide (Fig. 3).

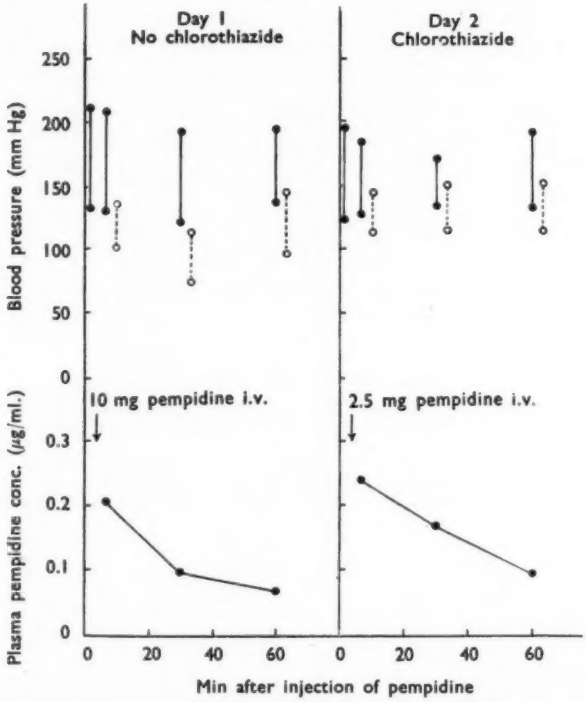

Fig. 3. Effect of chlorothiazide on the plasma pempidine concentration and the hypotensive response after an intravenous injection of pempidine. Blood pressures (●——● lying and ○- - -○ standing) and plasma pempidine concentrations were measured for 1 hr after injection of pempidine on Day 1 and again on Day 2 when the patient had been premedicated with chlorothiazide. Note that 10 mg of pempidine on Day 1 produced a larger hypotensive response but lower plasma pempidine concentrations than 2.5 mg on Day 2.

Intravenous pempidine and diuretics other than chlorothiazide. Patients given intramuscular mersalyl before intravenous pempidine showed an increase in both plasma pempidine concentration and hypotensive response. However, the effect on the plasma pempidine concentration was neither so great nor so prolonged as with chlorothiazide and was absent 3 days afterwards. The effect of mersalyl on the red cell/plasma distribution of pempidine is indicated in Table 3. Whereas when pempidine was given alone the distribution ratio decreased during the hour following the injection from about 2 to approximately 1.3 (see Table 2), a constant

value approximately 1.0 was obtained after the patient had been premedicated with intramuscular mersalyl (2 ml. mersalyl injection *B.P.* daily).

Acetazoleamide had no effect either on the pempidine concentration in plasma or on the hypotensive response.

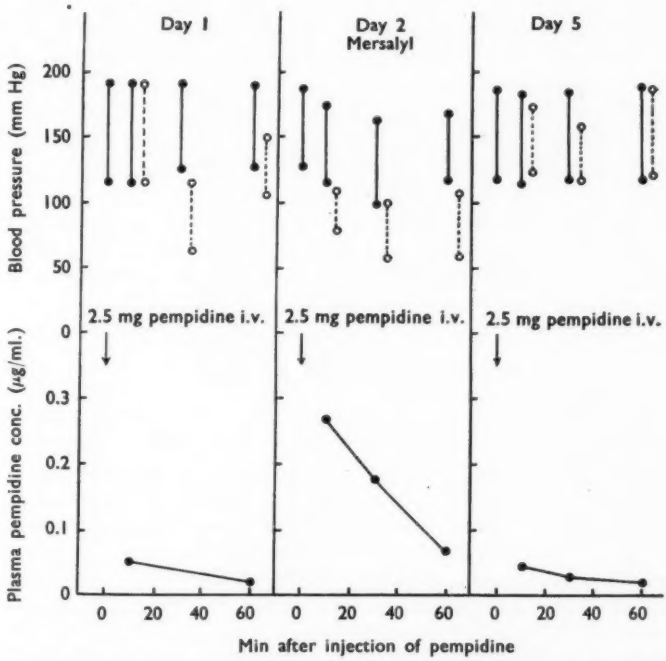

Fig. 4. Effect of mersalyl on the plasma pempidine concentration and the hypotensive response after an intravenous injection of pempidine. Blood pressures (● —— ● lying and ○ - - - ○ standing) and plasma pempidine concentrations were measured for 1 hr after an intravenous injection of pempidine (2.5 mg) on Days 1, 2 and 5. The patient received two intravenous injections of mersalyl before observations were made on Day 2. Note high plasma pempidine concentrations on Day 2.

TABLE 3

EFFECT OF INTRAMUSCULAR MERSALYL ON CONCENTRATIONS IN PLASMA AND RED CELLS OF PEMPIDINE INJECTED INTRAVENOUSLY

Concentrations of pempidine (μg/ml.) in plasma (P) and red cells (R.C.), and their ratio, for 1 hr after intravenous injection of the drug (2·5 mg). The patient was premedicated with mersalyl injection *B.P.* intramuscularly (2 ml. daily)

Time after pempidine injection (min)	P	R.C.	R.C./P
10	0·193	0·188	0·98
30	0·148	0·143	0·97
70	0·094	0·094	1·00

Oral pempidine and intravenous chlorothiazide. In each of 9 patients having maintenance treatment with oral pempidine the intravenous injection of 100 to 500 mg chlorothiazide produced within 1 hr a sharp rise in the plasma pempidine concentration. In some patients the increase was as much as 5-fold, without significant change in the postural fall in blood pressure (Fig. 5).

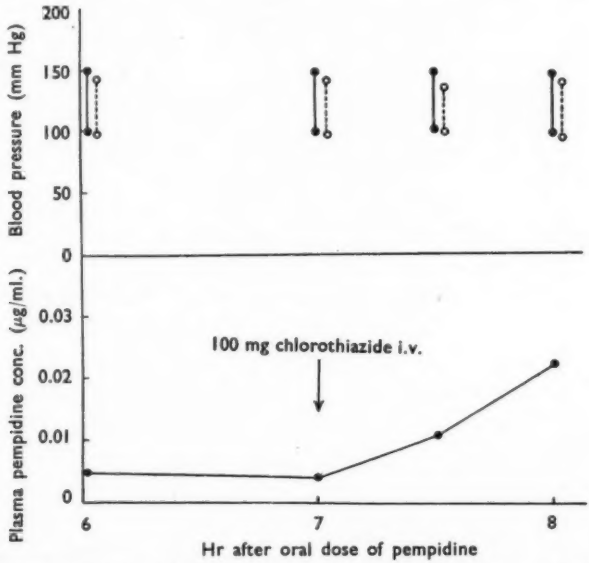

Fig. 5. Effect of intravenous chlorothiazide on the hypotensive response and plasma pempidine concentration after an oral dose of pempidine. 100 mg of chlorothiazide was injected 7 hr after 2.5 mg of pempidine had been given orally. The plasma pempidine concentration increased sharply without significant change in the postural fall in blood pressure (blood pressure standing ○ - - - ○ and lying ● —— ●).

TABLE 4

EFFECT OF INTRAVENOUS CHLOROTHIAZIDE ON CONCENTRATIONS IN PLASMA AND RED CELLS OF PEMPIDINE INJECTED INTRAVENOUSLY

Concentrations of pempidine (μg/ml.) in plasma (P) and red cells (R.C.), and their ratio, for 1 hr after intravenous injection of the drug (2·5 mg). Pempidine was given (i) alone, (ii) mixed with chlorothiazide, (iii) simultaneously with intravenous injection of chlorothiazide, (iv) to a patient premedicated with chlorothiazide

Time after pempidine injection (min)	No chlorothiazide			Chlorothiazide + pempidine (mixed)			Chlorothiazide and pempidine (simultaneously)			Premedication with chlorothiazide		
	P	R.C.	R.C./P	P	R.C.	R.C./P	P	R.C.	R.C./P	P	R.C.	R.C./P
5	0·040	—	—	0·060	—	—	—	—	—	0·328	—	—
10	—	—	—	—	—	—	0·044	0·076	1·73	0·258	0·180	0·702
30	0·030	—	—	0·041	—	—	0·035	0·036	1·03	0·202	0·139	0·698
60	0·022	—	—	0·022	—	—	0·028	0·018	0·64	0·134	0·094	0·689

Intravenous pempidine and intravenous chlorothiazide. The results of experiments in which both pempidine and chlorothiazide were administered intravenously are shown in Table 4. Plasma concentrations of pempidine for 1 hr after the injection of 2.5 mg intravenously were not significantly different if (*a*) no chlorothiazide was given, (*b*) chlorothiazide and pempidine were mixed in the syringe, (*c*) chlorothiazide and pempidine were injected simultaneously but separately. However, high plasma pempidine concentrations were obtained when the patient had received chlorothiazide intravenously 1 hr before the injection of pempidine. In this case the plasma pempidine concentrations and red cell/plasma distribution ratios were very similar to those obtained after oral premedication with chlorothiazide (see Table 2). The red cell/plasma ratios observed when chlorothiazide and pempidine were injected simultaneously showed a steady decline from 1.73 to 0.64 during the first hour, although the plasma pempidine concentrations were close to those obtained in similar studies without chlorothiazide. This suggested that the effect of chlorothiazide was not produced at once but developed relatively slowly. In subsequent studies, therefore, premedication with chlorothiazide was always carried out 1 hr before administering pempidine.

Renal excretion of pempidine

Preliminary experiments in which pempidine alone was infused revealed that after the plasma pempidine concentration became constant 2 hr might elapse before a steady rate of excretion was attained. We did not pursue this point further but made measurements of renal clearance only after the plasma pempidine concentration had been steady for at least 2 hr. Little change occurred in the excretion of pempidine when chlorothiazide was infused, but the calculated renal clearance fell to about one-third (Table 5). This decrease in renal clearance, which was not accompanied by a commensurate alteration in the renal excretion, was caused mainly by the 3-fold rise in the plasma pempidine concentration. Infusions of chlorothiazide raised the *p*H of urine, and, although this could be overcome by administering ammonium chloride orally, the renal pempidine clearance decreased as before (Fig. 6).

TABLE 5

EFFECT OF CHLOROTHIAZIDE ON THE RENAL CLEARANCE OF PEMPIDINE

P=plasma concentration (μg/ml.); UV=renal excretion (μg/min)=urine concentration × volume of urine/min; UV/P=renal clearance (ml./min) of pempidine. Pempidine was infused uniformly (about 1 mg/hr) into the patient without chlorothiazide (left-hand columns) and with chlorothiazide, 40 to 100 mg/hr (right-hand columns)

	Control period			Chlorothiazide period		
Patient	P	UV	UV/P	P	UV	UV/P
1	0·057	19·6	336	0·148	19·5	132
2	0·066	8·21	124	0·139	8·1	58
3	0·080	34·66	433	0·145	58·5	403
4	0·055	22·9	418	0·120	12·8	107
5	0·060	12·01	260	0·160	19·07	119
6	0·043	8·25	192	0·091	4·06	45
7	0·036	18·75	521	0·076	9·0	118
8	0·046	14·0	304	0·120	19·8	165

In one patient the urinary *p*H was measured daily for a week, but a definite shift to the alkaline side could be detected only on the first day of chlorothiazide treatment. The effect on renal pempidine clearance of chlorothiazide was different from that of changes in urinary *p*H. The effect of *p*H was studied by infusing sodium bicarbonate and it was generally small and rather variable. A rise in *p*H produced a lower clearance in 6 patients, little change in one and an increase in

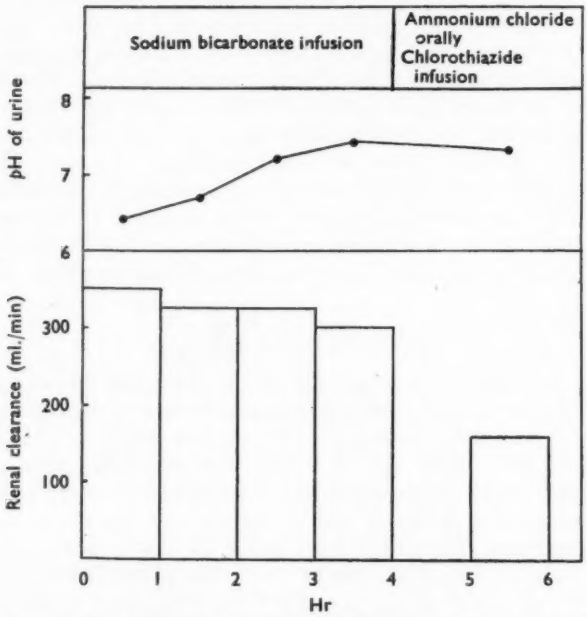

Fig. 6. Effect of chlorothiazide on the renal clearance of pempidine. Pempidine and chlorothiazide were infused at a steady rate (for details see text) and the urinary *p*H was controlled with sodium bicarbonate (intravenous) or ammonium chloride (oral).

another. With one exception the renal excretion of pempidine was less in alkaline urine (Table 6). For a change in urinary *p*H of (say) 6 to 7 the effect on renal pempidine clearance was often quite small ; for a similar *p*H change brought about by chlorothiazide, however, a much lower renal clearance was obtained. Chlorothiazide reduced the renal clearance of pempidine by raising the plasma concentration of the drug with only minor changes in the excretion. An alteration in urinary *p*H, however, may reduce the renal clearance by lowering the excretion without affecting the plasma pempidine concentration.

Distribution of pempidine in rat tissues

The effect of chlorothiazide on the distribution of pempidine in rat tissues is shown in Table 7. Results of duplicate experiments in which pempidine was administered orally to rats both without and with chlorothiazide pretreatment are recorded as well as mean values. Chlorothiazide produced a 3-fold increase in the plasma

TABLE 6

EFFECT OF URINARY *p*H ON THE RENAL CLEARANCE OF PEMPIDINE

Urinary *p*H, plasma pempidine concentration (μg/ml.), renal pempidine clearance (ml./min) and renal pempidine excretion (mg/hr) measured during uniform infusion of pempidine (1 mg/hr). Left-hand columns for initial period, right-hand columns after infusing sodium bicarbonate (20 to 100 mEq/hr) to raise *p*H of urine

	Initial period				After infusing sodium bicarbonate			
Patient	Plasma concentration	Urinary *p*H	Renal clearance	Renal excretion	Plasma concentration	Urinary *p*H	Renal clearance	Renal excretion
1	0·043	5·0	337	0·870	0·045	6·8	79	0·213
2		5·6	246			6·6	258	
3	0·043	6·3	332	0·086	0·050	7·1	242	0·073
4		5·2	433			6·4	403	
5		6·2	336			6·5	182	
6	0·046	5·3	279	0·770	0·048	6·7	307	0·884
7	0·060	6·5	288	1·02	0·060	7·9	200	0·721
8	0·048	6·5	330	0·95	0·046	7·4	304	0·840

TABLE 7

PEMPIDINE CONCENTRATIONS IN RAT TISSUES

Concentrations of pempidine (μg/g) in tissues of rats after administration of the drug (1 mg/kg) by stomach tube. The figures on the left of each column represent the results of two experiments on groups of 5 rats, those on the right represent the mean values. Left-hand column: Rats not pretreated with chlorothirazide. Right-hand column: Rats pretreated with chlorothiazide (5 mg/kg) 7, 4 and 1 hr before pempidine

Tissue	μg/g without chlorothiazide	μg/g with chlorothiazide
Plasma	1·38, 1·44 } 1·41	4·31, 4·06 } 4·18
Red cells	2·11, 2·32 } 2·22	3·14, 2·75 } 2·99
Spleen	1·41, 1·46 } 1·44	0·47, 0·51 } 0·49
Kidney	1·95, 2·18 } 2·06	2·54, 1·96 } 2·25
Liver	1·48, 1·38 } 1·43	1·48, 1·58 } 1·53
Lung	0·25, 0·42 } 0·34	0·26, 0·21 } 0·24
Brain	0·25, 0·36 } 0·30	0·24, 0·52 } 0·38
Muscle	0·16, 0·20 } 0·18	0·09, 0·14 } 0·12

pempidine concentration, a smaller rise also being obtained with red cells. The amount of pempidine in spleen was much lower after administration of chlorothiazide, but changes in other tissues were relatively small and are probably not significant because the results represent only 10 animals.

Distribution of pempidine between red cells and plasma in vitro

When the distribution of pempidine between red cells and plasma was studied *in vitro* wholly unexpected results were obtained. A known amount of pempidine was added to fresh whole blood, maintained at 37° C and gently stirred. Aliquots

were withdrawn at intervals and the pempidine content of the plasma and the red cells measured. During the first 3 min of the experiment the plasma pempidine concentration decreased rapidly and thereafter increased steadily. The corresponding concentrations of pempidine in the red cells at first increased rapidly and then decreased so that the pempidine content of the whole blood was constant within

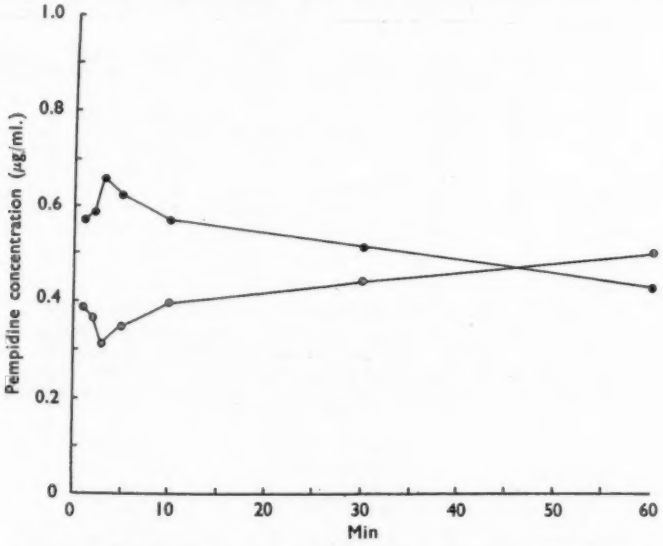

Fig. 7. Concentrations of pempidine in plasma and red cells *in vitro*. Pempidine was added to heparinized whole blood and the amount of pempidine in the plasma (○ —— ○) and red cells (● —— ●) measured at intervals for 1 hr.

the limits of experimental error (Fig. 7). During the first 45 min the concentration of pempidine was higher in red cells than in plasma, but thereafter the plasma concentration became the greater. Almost identical results were obtained with blood which had been incubated for 1 hr at 37° C before the addition of pempidine. In the presence of chlorothiazide, however, an equilibrium was established before the first measurement could be made (1 min) and no change in the pempidine concentration of either red cells or plasma was observed during the course of 1 hr. At all times the plasma pempidine concentration was greater than that in red cells (Fig. 8). Changes with time in the ratio $\frac{\text{red-cell pempidine concentration}}{\text{plasma pempidine concentration}}$ are shown in Fig. 9. Thus, in the absence of chlorothiazide the red-cell/plasma ratio passed through a well-defined maximum, whereas in the presence of chlorothiazide it showed no such variation but attained a steady value almost instantaneously. Within the limits of experimental error the same distribution ratio of pempidine between red cells and plasma was found (*a*) with blood to which chlorothiazide had been added *in vitro* (0.67); (*b*) with blood taken from a patient who had been premedicated with 500 mg of intravenous chlorothiazide 1 hr before (0.69); (*c*) *in vivo*

after intravenous injection of pempidine in patients premedicated with chlorothiazide (0.69). When blood was incubated with mersalyl *in vitro* before the addition of pempidine the distribution ratio of pempidine between red cells and plasma (1.0) did not change with time and, moreover, was the same as that found *in vivo* after intramuscular injections of mersalyl (see Table 3).

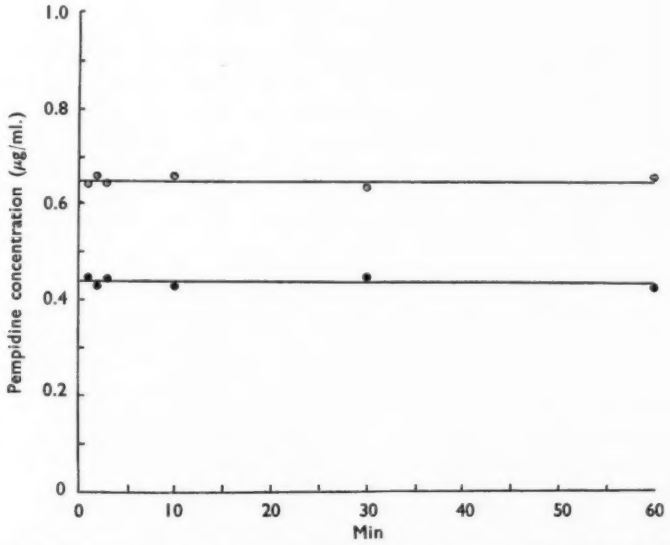

Fig. 8. Effect of chlorothiazide on concentrations of pempidine in plasma and red cells *in vitro*. Pempidine was added to heparinized whole blood containing chlorothiazide added *in vitro* and the concentrations of pempidine in the plasma (○ —— ○) and red cells (● —— ●) measured at intervals for 1 hr.

TABLE 8

THE EFFECT OF CHLOROTHIAZIDE ON THE BINDING OF PEMPIDINE TO SERUM ALBUMIN

Free and protein-bound pempidine measured by equilibrium dialysis. Initial concentration of pempidine (outer compartment) 1 μg/ml. Initial concentration of chlorothiazide (inner compartment) 2·5 to 50·0 μg/ml. Concentration of serum albumin (inner compartment) 1%

Pempidine (μg/ml.)				Chlorothiazide (μg/ml.)			
Total	Free	Bound	Bound/ Total	Total	Free	Bound	Bound/ Total
0·92	0·77	0·15	0·16	1·3	0·3	1·0	0·77
1·04	0·74	0·30	0·29	2·6	0·6	2·0	0·77
1·08	0·73	0·35	0·32	3·1	1·1	2·0	0·65
1·08	0·73	0·35	0·32	5·32	1·17	4·15	0·78
1·16	0·71	0·45	0·39	7·4	1·9	5·5	0·74
1·28	0·68	0·60	0·47	10·6	2·35	8·25	0·78
1·40	0·65	0·75	0·54	14·6	3·85	10·75	0·74
1·52	0·62	0·90	0·59	19·2	5·2	14·0	0·73
1·76	0·56	1·20	0·68	24·6	6·35	18·25	0·74

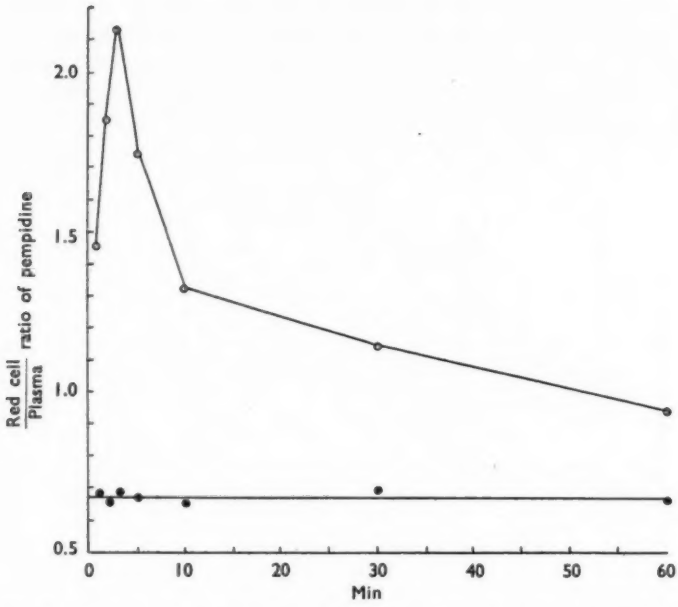

Fig. 9. Distribution ratio of pempidine *in vitro* between red cells and plasma, with (●—●) and without (○—○) chlorothiazide. The chlorothiazide was added *in vitro* to heparinized whole blood.

TABLE 9

THE EFFECT OF CHLOROTHIAZIDE ON THE BINDING OF PEMPIDINE TO SERUM ALBUMIN

Free and protein-bound pempidine measured by equilibrium dialysis. Initial concentration of pempidine (outer compartment) 0 to 1·0 μg/ml. Initial concentration of chlorothiazide (inner compartment) 50 μg/ml. concentration of serum albumin (inner compartment) 1%

Pempidine (μg/ml.)				Chlorothiazide (μg/ml.)			
Total	Free	Bound	Bound/ Total	Total	Free	Bound	Bound/ Total
0·212	0·047	0·165	0·78	23·8	6·55	17·25	0·73
0·400	0·100	0·300	0·75	25·0	6·26	18·74	0·75
0·588	0·153	0·435	0·74	24·0	6·50	17·50	0·73
0·784	0·204	0·580	0·74	23·4	6·65	16·75	0·72
1·000	0·250	0·750	0·75	23·6	6·61	16·99	0·72
1·152	0·312	0·840	0·73	23·9	6·56	17·34	0·73
1·300	0·375	0·925	0·71	24·6	6·35	18·25	0·74
1·408	0·448	0·960	0·68	24·1	6·48	17·62	0·73
1·548	0·513	1·035	0·67	25·8	6·05	19·75	0·77
1·680	0·580	1·100	0·66	26·2	5·95	20·25	0·77

Binding of pempidine to serum albumin in vitro

Equilibrium dialysis experiments carried out in the absence of protein showed that neither pempidine nor chlorothiazide was adsorbed on the dialysis sac. In

every case the observed changes in concentration were due solely to diffusion processes. Pempidine alone (that is, in the absence of chlorothiazide) did not interact with serum albumin to any measurable extent. On the other hand, chlorothiazide alone (that is, in the absence of pempidine) was extensively bound, and the present results agree with the findings of Baer and his colleagues (1959). The results of the two most important series of experiments are shown in Tables 8 and 9.

DISCUSSION

In treating severe hypertension it is often necessary to administer diuretics, and many workers consider that chlorothiazide, when used in this way, potentiates the hypotensive action of ganglion-blocking agents. This is so with pempidine although the effect is quite small. Our previous studies showed that the fall in blood-pressure after giving pempidine orally was related to the plasma concentration of the drug. This correlation no longer holds in the presence of chlorothiazide, which may almost treble the plasma pempidine concentration with only a slight increase in the hypotension.

The increased response to pempidine after the administration of chlorothiazide might be attributed to an inherent hypotensive activity of chlorothiazide itself, but in our experience this cannot be convincingly demonstrated. Chlorothiazide does, however, potentiate the action of ganglion-blocking agents by reducing the plasma volume, although this effect combined with the very high plasma pempidine concentrations now reported should produce a much larger fall in blood-pressure than was actually observed. One must be cautious in comparing results of intravenous studies with those obtained from patients having regular daily doses of pempidine and chlorothiazide by mouth. Sustained plasma pempidine concentrations could conceivably have a different effect from the transient elevation after intravenous injection, while repeated doses of chlorothiazide reduce plasma volume and deplete body sodium. The experiments illustrated in Fig. 3 show that a high plasma pempidine concentration achieved with the aid of chlorothiazide may produce a smaller hypotensive effect than that caused by an equal concentration obtained merely by increasing the dose of pempidine. This implies that chlorothiazide may increase the plasma concentration of pempidine and also modify its pharmacological activity.

High plasma pempidine concentrations obtained by administering chlorothiazide suggest an alteration in renal clearance. Harington & Kincaid-Smith (1958) suggested that chlorothiazide reduced the renal excretion of mecamylamine by inhibiting carbonic anhydrase activity, so that the urine became more alkaline. The hypothesis is not entirely satisfactory because the inhibition of carbonic anhydrase is only transitory and, moreover, acetazoleamide has little effect on the hypotensive effect of mecamylamine although it is a powerful inhibitor of the enzyme (Bayliss, Fleming & Rees, 1958). We do not consider this mechanism to be of importance in the case of pempidine. Nevertheless, chlorothiazide does lower the renal clearance (though not the excretion) of pempidine by more than 50%. This cannot be attributed to an increase in the urinary *p*H because an alkaline urine obtained by infusions of sodium bicarbonate lowered the renal clearance of

pempidine by only about 15%. The observed reduction in renal clearance of pempidine brought about by chlorothiazide must therefore depend on the increased plasma pempidine concentration. During the infusion experiments the renal pempidine clearance increased for some hours after a steady plasma concentration of the drug was established. This probably means that pempidine passed initially into the kidney parenchyma until an equilibrium was set up ; at this point the renal clearance became constant.

If chlorothiazide increased the plasma pempidine concentration by changing the tissue distribution, pempidine would presumably be transferred from intracellular depots to the extracellular fluid. The experiments with rats suggest that this does not happen, because chlorothiazide causes no major redistribution of pempidine among the tissues. The pempidine content of spleen is decreased to some extent, but clearly the amount of drug released is insufficient to account for the very high concentrations of pempidine in the plasma. The most drastic changes in pempidine distribution are those in red cells and plasma.

We consider that a reasonable explanation of our findings in hypertensive patients can be gven in terms of a pempidine/protein interaction induced by chlorothiazide. *In vitro* experiments, using equilibrium dialysis techniques, show that although in solution pempidine does not normally interact with serum albumin to any measurable extent, a pempidine/albumin complex of some kind is formed in the presence of chlorothiazide. The degree of binding can be extensive, depending on the amounts of pempidine and chlorothiazide present. It is reasonable to suppose that similar interactions occur *in vivo* and might involve other proteins as well as albumin.

The high concentrations of pempidine in plasma after the administration of chlorothiazide are due to the presence of a " bound " fraction of the pempidine closely associated with the plasma proteins and producing no hypotensive effect, in addition to the " free " pempidine, which is pharmacologically active. The " bound " pempidine does not cause hypotension because, being bound to protein, it cannot penetrate to the site of action. Moreover, it is not filtered at the glomerulus and the overall renal clearance is therefore reduced.

High plasma pempidine concentrations may be detected chemically although only part of the drug is in a form which can exert a physiological effect. This is consistent with the observation that plasma pempidine concentrations increase rapidly when patients on maintenance treatment are given intravenous injections of chlorothiazide although no change in the blood pressure occurs. The normal passage of drug from intracellular stores to extracellular fluid continues although the pempidine is no longer excreted at the former rate but is instead bound to plasma protein in an inactive form. Consequently, the blood-pressure does not fall significantly although the plasma pempidine concentration may be greatly increased. Protein-binding can account for the high plasma pempidine concentrations and reduced renal clearance which occur after giving chlorothiazide, but other factors also seem to be involved. It is known, for instance, that chlorothiazide is excreted within 18 hr (Ford, Moyer & Spurr, 1957) and it is by no means clear how chlorothiazide continues to affect plasma pempidine concentrations after 3 days.

The striking changes that chlorothiazide produces in the red-cell/plasma distribution ratio of pempidine can also be partly attributed to the formation of a complex by pempidine and plasma proteins. *In vivo* the stable ratio between red cells and plasma is about 1.2:1, but after chlorothiazide it falls to about 0.7:1. The transfer of pempidine from red cells into plasma is presumably a result of binding to plasma proteins and reflects a lower "free" pempidine concentration in plasma. This hypothesis explains neither the abolition by chlorothiazide of the changing red-cell/plasma ratio in the first hour after the addition of pempidine *in vivo* or *in vitro*, nor the red-cell/plasma ratio in patients given intravenous pempidine after premedication with chlorothiazide. It is interesting to note, however, that the red-cell/plasma ratio for pempidine in the presence of chlorothiazide ($\sim$0.7) is the same as found by Baer, Leidy & Brooks (1957) for the partition of chlorothiazide between red cells and plasma. However, because at present we cannot explain the normal change in the distribution of pempidine between cells and plasma during the first hour after mixing, it is difficult even to speculate about the way in which chlorothiazide modifies it. At first sight the distribution in the pempidine injection studies *in vivo* may seem compatible with the hypothesis of plasma-protein-binding, but a number of difficulties arise. For example, if the "free" fraction of pempidine were cleared from the plasma at the normal rate for pempidine without chlorothiazide, a low "free" concentration would be expected. Furthermore, because the red cells are presumably in equilibrium with the "free" pempidine fraction their pempidine content should also not only be low but should fall rapidly. In fact, this does not happen in practice. It seems probable that just as chlorothiazide promotes interaction of pempidine and plasma protein so it may modify the affinity of red cells for the drug; however, we have no direct experimental evidence on this point.

The interactions of pempidine and chlorothiazide with proteins and with organized structures such as red cells are of great interest. The results reported in this paper raise a number of important matters which, however, cannot be discussed at length here. For example, the nature of the pempidine/protein complex is quite unknown and merits further study. Again, chlorothiazide and pempidine are distributed between red cells and plasma in almost the same ratio, but the significance of this is not clear at present. The delay in onset of the chlorothiazide effect (see Table 4) is curious. When pempidine and chlorothiazide were injected simultaneously normal plasma pempidine concentrations were obtained, but the red-cell/plasma distribution ratio of pempidine decreased during the first hour to the value observed in patients premedicated with chlorothiazide. This suggests that pempidine had already left the blood before it could be bound to plasma protein by the action of chlorothiazide. This delay is unexplained. Without doubt, however, the most perplexing observation is the rapid transport of pempidine into and out of red cells that occurs immediately after mixing. It is difficult to formulate a satisfactory mechanism, especially as chlorothiazide appears able either to inhibit the process or to increase its rate to such an extent that it is completed instantaneously. We do not at present know if these phenomena have a common basis, but it is tempting to speculate whether chlorothiazide can modify the surfaces of proteins and hence change their adsorptive properties. This would possibly enable the protein/pempidine complex to persist even after the chlorothiazide had been removed. Unfortunately, we have at present

no experimental evidence to support this hypothesis, but an examination by electrophoresis or ultracentrifugation of various proteins and cells before and after treatment with chlorothiazide might be rewarding.

We gratefully acknowledge the valuable technical assistance given by Miss M. I. Tinson and Mr A. F. Ivens who carried out the dialysis experiments and fluorescence measurements respectively. Thanks are due also to Dr H. W. Reading for performing the rat experiments and to Dr H. Campbell and Professor J. McMichael, F.R.S., for their continued interest and support.

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CENTRALLY ACTING MUSCLE RELAXANTS IN TETANUS

BY

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The anti-tetanus activity of a number of phenothiazine derivatives and other centrally acting muscle relaxants, such as mephenesin, dicyclopropyl ketoxime, 2-amino-6-methylbenzothiazole and meprobamate, has been determined in rabbits with experimental local tetanus. Structure–activity relationships were obtained for the phenothiazine derivatives and their anti-tetanus activity correlated with other central and peripheral properties. Both dicyclopropyl ketoxime and 2-amino-6-methylbenzothiazole were twice as active as mephenesin. Meprobamate does not appear to be primarily a muscle relaxant of the mephenesin type.

Tetanus may be suppressed by drugs from a variety of chemical groups having widely differing types of activity. Two drugs that have had considerable clinical use are the centrally acting muscle relaxants, mephenesin and chlorpromazine. They both reduce muscle spasm and control convulsions without causing loss of consciousness; although when tested against local tetanus in the rabbit they have distinctly different types of activity (Webster, unpublished).

In the present work further compounds, either similar in structure to chlorpromazine, that is, other phenothiazine derivatives, or similar in action to mephenesin, have been tested and their relative activities determined. Some preliminary results have been published previously (Laurence & Webster, 1958b).

Derivatives of phenothiazine (Table 1) are obtained either by modifying the aminopropyl side-chain (R_1) or by introducing various radicals into position 2 of the phenothiazine nucleus (R_2). The effect of these changes has been assessed by testing a range of 8 derivatives.

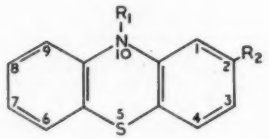
Promethazine, the basic member of the group, is the 2-dimethylaminopropyl derivative of phenothiazine. Replacement of its side-chain by the non-ramified 3-dimethylaminopropyl radical gives promazine. The effect on anti-tetanus activity of this change and of introducing a chlorine atom or acetyl group into position 2 of promazine has been determined in a quantitative assay of promethazine, promazine, chlorpromazine and acepromazine.

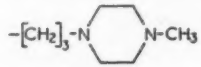
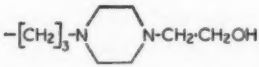
Further compounds tested include trimeprazine and methotrimeprazine, in which the 3-aminopropyl side-chain has been further modified by the addition of a methyl group to the second carbon atom, and perphenazine and prochlorperazine, in both of which a piperazine ring has been incorporated in the side-chain. Other derivatives were tested, but their potency has not been exactly determined.

* Stothert Research Fellow of the Royal Society.

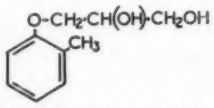
TABLE 1

THE STRUCTURAL FORMULAE OF SOME PHENOTHIAZINES TESTED FOR ANTI-TETANUS ACTIVITY

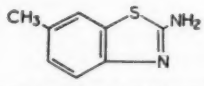


R_1	R_2			
	H	–Cl	$-OCH_3$	$-CO.CH_3$
–H	Phenothiazine			
$-CH_2.CH(CH_3).N(CH_3)_2$	Promethazine			
$-[CH_2]_3.N(CH_3)_2$	Promazine	Chlorpromazine		Acepromazine
$-CH_2.CH(CH_3).CH_2.N(CH_3)_2$	Trimeprazine		Methotrimeprazine	
		Prochlorperazine		
		Perphenazine		

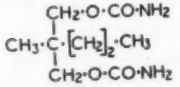
If the rapid and complete abolition of tetanus produced by mephenesin results from its depressant action on spinal interneurones, then other centrally acting muscle relaxants, which are similar in action to mephenesin, may also be effective against tetanus. Three such compounds, whose formulae are shown in Fig. 1, have been tested. They are meprobamate, 2-amino-6-methylbenzothiazole and dicyclopropyl ketoxime.



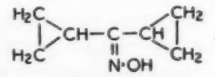
3-(o-Tolyloxy)propane-1,2-diol
Mephenesin



2-Amino-6-methylbenzothiazole
SKF 1045 A



2,2-Di(carbamoyloxymethyl)pentane
Meprobamate



Dicyclopropyl ketoxime
MR 710

Fig. 1. Structural formulae of some centrally acting muscle relaxants.

One of the main disadvantages of mephenesin is that it is short-acting due to the rapid oxidation of its hydroxyl group (Riley & Berger, 1949). Attempts have been made to produce longer-acting compounds by blocking this group. A series of

derivatives was prepared by Berger (1952) in which the hydroxyl group was transformed to a carbamoyloxy group, and of these 2,2-di(carbamoyloxymethyl)pentane (meprobamate) was found to possess central actions similar to mephenesin and to be more potent and longer-acting. The induction period and duration of paralysis produced by meprobamate in mice are both ten times as long as the corresponding periods for mephenesin. It is more active than mephenesin against leptazol and electroshock-induced convulsions, but only equally active against strychnine convulsions (Berger, 1954). Abolition of the flexor, crossed extensor and linguomandibular reflexes in the cat is obtained with 20 to 40 mg/kg compared with half this dose of mephenesin. This difference in potency on spinal reflexes has also been observed by Pfeiffer, Riopelle, Smith, Jenney & Williams (1957). Meprobamate has had wide use as a tranquillizing agent, and although it does not produce any marked change in the electroencephalogram it is known to synchronize activity recorded from the thalamus (Berger, Campbell, Hendley, Ludwig & Lynes, 1957; Hendley, Lynes & Berger, 1955). Anderson & Kjaer (1958) and Perlstein (1959) have used it to relieve muscle spasm in human tetanus.

The compound 2-amino-6-methylbenzothiazole is chemically related to benzimidazole, which produces muscular relaxation, antagonizes strychnine convulsions, abolishes multisynaptic reflexes without affecting monosynaptic transmission and reduces rigidity in decerebrate cats (Goodman, Gilman & Hart, 1943; Goodman & Hart, 1944) but is less active than mephenesin. A series of benzazole derivatives were compared by Domino, Unna & Kerwin (1952) for their ability to produce paralysis in mice, rabbits and dogs. Most of them produced a mixture of stimulating and depressant effects, but the substituted 2-aminobenzothiazoles were particularly potent paralysing agents and 2-amino-6-methylbenzothiazole especially free of stimulating effects. This compound was shown by Funderburk, King, Domino & Unna (1953), using myographic and ventral root recording, to abolish polysynaptic reflexes and also the facilitating and inhibitory effects of bulbar stimulation on spinal activity. It also reduced decerebrate rigidity, and spasticity caused by anoxia of the spinal cord. In all these actions it is twice as potent as mephenesin and longer-acting. Doses that depressed the spinal cord had no effect on the electroencephalogram. The effects on spinal activity were confirmed by King & Unna (1954).

Dicyclopropyl ketoxime is a new spinal depressant, more active than mephenesin, which has been used successfully in the control of tetanus spasms and convulsions (Richards, Blockus, Elam & Perlstein, 1958).

METHODS

Local tetanus was induced in rabbits by the injection of 625 mouse minimal lethal doses (MLD) of toxin into the gastrocnemius muscle of one hind limb of the rabbit and activated by a standard afferent stimulus (Laurence & Webster, 1958a). Resulting muscle activity was measured by quantitative electromyography and the level of tetanus expressed in terms of units of activity per minute (Laurence & Webster, 1958b). Percentage reductions were calculated for the 30 min following each injection. All drugs were injected intravenously and all were in aqueous solution apart from dicyclopropyl ketoxime and meprobamate, which were prepared in aqueous propylene glycol.

RESULTS

Phenothiazine derivatives

The following phenothiazines were selected for accurate assay on 7 rabbits, all of which received two doses of each compound:

Promethazine hydrochloride
10-(2-dimethylaminopropyl)phenothiazine hydrochloride.

Promazine hydrochloride
10-(3-dimethylaminopropyl)phenothiazine hydrochloride.

Chlorpromazine hydrochloride
2-chloro-10-(3-dimethylaminopropyl)phenothiazine hydrochloride.

Acepromazine maleate
2-acetyl-10-(3-dimethylaminopropyl)phenothiazine maleate.

The % reduction produced by each dose is given in Table 2 together with the dose producing a 50% reduction of activity calculated graphically from the mean results. The activity ratio of each compound relative to chlorpromazine, calculated

TABLE 2

REDUCTIONS IN TETANUS ACTIVITY RECORDED IN THE ASSAY OF THE ANTI-TETANUS ACTIVITY OF PROMETHAZINE, PROMAZINE, CHLORPROMAZINE AND ACEPROMAZINE

The 50% reduction doses were determined graphically from the regression line obtained from the plot of mean results. Deviations from parallelism were calculated in comparison with chlorpromazine. Reductions were calculated for the 30 min after each injection

	% reduction							
	Promethazine		Promazine		Chlorpromazine		Acepromazine	
Dose mg/kg:	0·5	1·0	0·25	0·50	0·125	0·25	0·0125	0·025
Rabbit								
1	34	65	22	72	17	50	44	72
2	37	64	17	17	28	51	0	14
3	18	78	12	29	45	35	23	61
4	61	89	46	59	26	69	28	78
5	50	81	41	61	34	76	56	64
6	58	71	21	72	53	73	56	75
7	44	86	63	90	36	70	60	58
Mean	43	76	31	57	34	60	38	60
s.d.	14·8	9·95	22·6	25·7	12·6	15·3	22·2	21·7
Dose causing 50% reduction (base mg/kg)	0·536		0·384		0·18		0·019	
Activity ratio	0·34		0·47		1·0		9·4	
Regression								
t	4·92		2·14		3·6		1·89	
P	0·001		0·1–0·05		0·1–0·001		0·1–0·05	
Deviation from parallel *t*	0·77		0·09		—		0·4	

from the dose causing 50% reduction, together with the "t" value for significance of regression and deviation from parallelism, is also given. Acepromazine was found to be 9.4 times as active as chlorpromazine. Promazine (0.46 times) and promethazine (0.34 times) were both less active.

The "t" value for regression is highly significant for promethazine and chlorpromazine but not significant for promazine and acepromazine. The results obtained with the latter two drugs must therefore be viewed with caution. A reversal of response was obtained with two doses of acepromazine in one animal.

The following phenothiazines were also tested:

Trimeprazine tartrate
10-(3-dimethylamino-2-methylpropyl)phenothiazine tartrate.

Methotrimeprazine
10-(3-dimethylamino-2-methylpropyl)-2-methoxyphenothiazine.

Perphenazine
2-chloro-10-[3-{4-(2-hydroxyethyl)piperazin-1-yl} propyl]phenothiazine.

Percentage reductions in activity were recorded in a number of animals for varied doses of the above derivatives, injected without any systematic order. Each rabbit received at least one dose of chlorpromazine and one dose of another compound. The following simplified procedure was then adopted to determine anti-tetanus activity. The reductions in tetanus activity were averaged, each average being the mean of at least 4 observations. The final activity ratios were determined by assuming a regression for chlorpromazine similar to that found in the experiments summarized in Table 2, the justification for adopting this procedure being that the reductions in tetanus obtained with chorpromazine in the present series were similar to those in the earlier one. The activity ratios relative to chlorpromazine, obtained in this manner, were methotrimeprazine, 2.4; trimeprazine, 0.5; and perphenazine, 0.25.

Prochlorperazine methanesulphonate, 2-chloro-10-{3-(4-methylpiperazin-1-yl)-propyl}phenothiazine methanesulphonate, showed anti-tetanus activity only with doses in excess of 1 mg/kg and probably possesses 1/10th of the activity of chlorpromazine. A similar order of activity was obtained with pecazine [mepazine; 10-(1-methylpiperid-3-ylmethyl)phenothiazine].

In summary the following anti-tetanus activities were found. The bracketed values indicate approximate estimates.

Acepromazine	9.4	Promazine	0.46
Methotrimeprazine	(2.4)	Promethazine	0.34
Chlorpromazine	1.0	Perphenazine	(0.25)
Trimeprazine	(0.5)	Prochlorperazine	(0.1)

Compounds with mephenesin-like action

The effects of mephenesin, meprobamate, dicyclopropyl ketoxime and 2-amino-6-methylbenzothiazole were tested on a number of rabbits. Whenever possible more than one drug was given to each animal, but due to unaccountable fluctuations in the persistence of local tetanus during the experiment this was not always possible. Recorded reductions in activity are given in Table 3. Although these results do not lend themselves to statistical analysis, the best estimate of relative activity was

TABLE 3

PERCENTAGE REDUCTIONS IN TETANUS ACTIVITY PRODUCED BY SOME CENTRALLY ACTING MUSCLE RELAXANTS

Percentage reductions in tetanus activity were calculated for 20 min after each injection. The method of calculating relative activities is outlined in the text

	Mephenesin		2-Amino-6-methyl-benzothiazole		Dicyclopropyl ketoxime		Meprobamate	
Animal	Dose mg/kg	% reduction	Dose mg/kg	% reduction	Dose mg/kg	% reduction	Dose mg/kg	% reduction
1			20	61	20	75		
2	50	36	25	60	25	81		
3	50	69			25	84	30	42
4					25	42	30	13
5	50	92	25	92	25	69		
6			25	90				
7					25	76		
8	25	57					25	16
9	25	49					50	43
Activity ratio	1·0		2·6		2·3		0·43	

obtained by a method similar to that outlined in the preceding section except that the average regressions for mephenesin and meprobamate were used for the calculation of activity ratios.

Both dicyclopropyl ketoxime and 2-amino-6-methylbenzothiazole were over twice as active as mephenesin, but meprobamate showed less anti-tetanus activity at the dose used. The mean time courses of action of these drugs are plotted in Fig. 2.

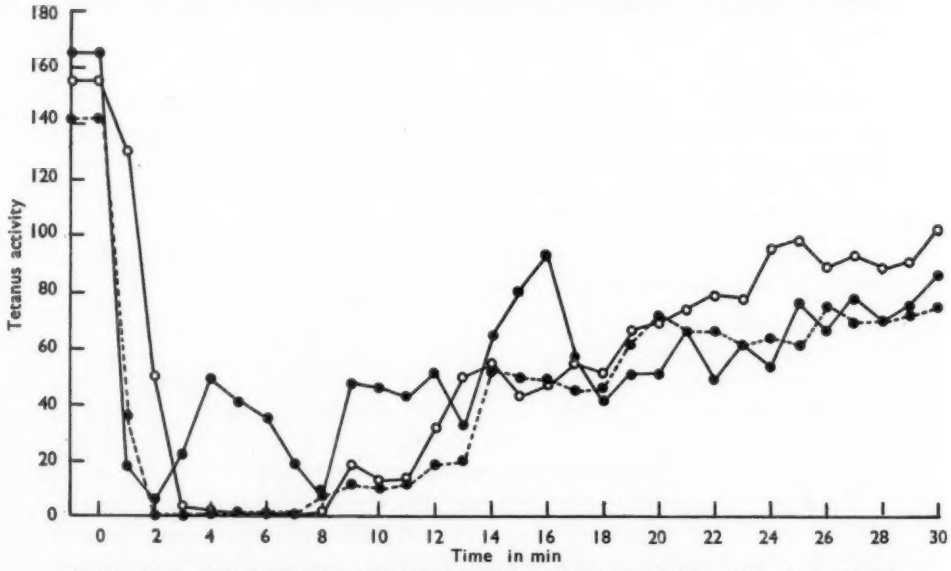


Fig. 2. Mean time course of the anti-tetanus activity of mephenesin (●---●), dicyclopropyl ketoxime (●——●) and 2-amino-6-methyl-benzothiazole (○——○). Mean results for 4 animals are shown. All injections were performed at 0 min. Tetanus activity is expressed as the count of units of integrated electromyogram activity.

Although all compounds were basically similar in action, dicyclopropyl ketoxime did not produce the same depth of depression as mephenesin, and in each animal receiving this compound sudden bursts of activity lasting 2 to 3 min occurred between periods of complete abolition.

DISCUSSION

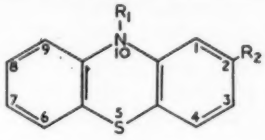
In these experiments phenothiazine derivatives with different side-chains and with various radicals attached to position 2 of the phenothiazine nucleus (Table 1) have been tested for anti-tetanus activity. The results obtained make it possible to draw certain conclusions regarding structure–activity relationships.

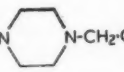
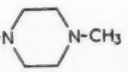
Representatives of five series of derivatives with different side-chains, promethazine, promazine, trimeprazine, prochlorperazine and perphenazine, have been investigated. The data are summarized in Table 4.

TABLE 4

THE EFFECT OF CHEMICAL STRUCTURE ON THE ANTI-TETANUS ACTIVITY OF SOME PHENOTHIAZINE DERIVATIVES RELATIVE TO CHLORPROMAZINE

The experimentally determined activity ratios are shown together in the square with each compound. The contribution of the side-chains to the activity ratios is given by the factors r_1 and r_2. The method of calculation of r_1 and r_2 is discussed in the text



R_1		R_2			
		—	–Cl	$-OCH_3$	$-CO.CH_3$
	r_1/r_2	0·46	1·0	2·2	9·4
$-CH_2.CH(CH_3).CH_2.N(CH_3)_2$	1·09	Trimeprazine 0·5		Metho-trimeprazine 2·4	
$-[CH_2]_3.N(CH_3)_2$	1·0	Promazine 0.46	Chlorpro-mazine 1·0		Acepro-mazine 9·4
$-CH_2.CH(CH_3).N(CH_3)_2$	0·716	Prometha-zine 0·33			
$-[CH_2]_3-N$ 	0·25		Perphenazine 0·25		
$-[CH_2]_3-N$ 	0·1		Prochlor-perazine 0.1		

This table contains activity ratios relative to chlorpromazine, of the anti-tetanus activity of a number of phenothiazine derivatives differing in respect of side-chains R_1 and R_2. The contributions r_1 and r_2 of each side-chain to the activity ratio have been calculated in such a way that $r_1 r_2$ = activity ratio. For example, to calculate the contributions of the side-chains in methotrimeprazine:

r_1 (trimeprazine side-chain R_1): $TP/PZ = 0.5/0.46 = 1.09$

r_2 (methoxyl side-chain R_2): $\frac{MT}{TP} \Big/ \frac{CP}{PZ} = \frac{2.4}{0.5} \Big/ \frac{1}{0.46} = 2.2$

Hence r_1r_2 (1.09) (2.2)=2.4=experimental activity ratio. TP=trimeprazine, PZ=promazine, MT=methotrimeprazine, CP=chlorpromazine.

The most active compounds are those with a 3-dimethylaminopropyl or a 3-dimethylamino-2-methylpropyl side-chain. The introduction of a piperazine ring reduces anti-tetanus activity considerably. The order of activity of the different side-chains is given in Table 4. With regard to the side-chain R_2 the acetyl and methoxyl derivatives are the most active. The order of potency of the side-chains R_2 is $COCH_3 : OCH_3 : Cl : H$ in the ratio 9.4 : 2.2 : 1.0 : 0.46.

It would be interesting to test the validity of the assumptions made in calculating the contributions of the two side-chains to anti-tetanus activity by testing compounds whose activity can be predicted from Table 4 by "completing a square." Thus the activity of methoxypromazine would be expected to be 2.2 and that of chlorpromethazine 0.72.

TABLE 5

A COMPARISON OF THE ANTI-TETANUS AND OTHER CENTRAL ACTIONS OF SOME PHENOTHIAZINE DERIVATIVES

The activity of each compound is expressed in relation to chlorpromazine. Some values were estimated from experimental results given in the papers referred to and are therefore only approximate

Derivative	Anti-tetanus activity	Depression of motor activity	Conditioned response	Anaesthetic potentiation	Anti-emetic activity	Reference
Acepromazine	9·4	1·66	—	—	—	(1)
		2·0	—	—	—	(2)
	—			6·0	2·0	(3)
Methotrimeprazine	2·4	2·5	2·0	4·0	0·9	(4)
Trimeprazine	0·5	0·66	0·6	0·8	0·33	(5)
Promazine	0·47	0·5				(6)
		0·5	0·5	0·5	0·2	(7)
Promethazine	0·34	0·05	0·5			(8)
				0·25		(9)
					0·1	(10)
Perphenazine	0·25	10·0	10·0	1·0		(6)
					24·0	(11)
Prochlorperazine	0·1	0·4	1·0	0·5	4·0	(12)

References: (1) Delay & Deniker (1957). (2) Wirth, Gässwald, Hörlein, Risse & Kreiskott (1958). (3) Mercier, Schmitt, Navarro, Gavend & Gavend (1957). (4) Courvoisier, Ducrot, Fournel & Julou (1957b, 1957c). (5) Courvoisier, Ducrot, Fournel & Julou (1958). (6) Arrigoni-Martelli & Kramer (1959). (7) Wirth (1958). (8) Fellows & Cook (1957). (9) Kopera & Armitage (1954). (10) Courvoisier, Fournel, Ducrot, Kolsky & Koetschet (1953). (11) Rosenkilde & Govier (1957). (12) Courvoisier, Ducrot, Fournel & Julou (1957a).

In an attempt to correlate anti-tetanus activity with some other property of the phenothiazine derivatives the literature on the pharmacology of the eight derivatives tested was reviewed. Amongst other indices of central nervous system activity, summarized in Table 5, only that for potentiation of anaesthesia appears to be correlated with anti-tetanus activity. Acepromazine is 6 times as active as chlorpromazine in this respect, which agrees reasonably with anti-tetanus activity (9.4 times), but perphenazine and prochlorperazine are relatively more active in prolonging anaesthesia than against tetanus.

From observations on anti-adrenaline, hypothermic and antihistamine activity, summarized in Table 6, it appears that there is a good correlation between anti-

TABLE 6

A COMPARISON OF THE ANTI-TETANUS, ANTI-ADRENALINE, ANTIHISTAMINE AND HYPOTHERMIC ACTIVITY OF SOME PHENOTHIAZINE DERIVATIVES

Derivative	Anti-tetanus activity	Anti-adrenaline activity	Anti-histamine activity	Hypo-thermic activity	Ref.
Acepromazine	9·4	10·0			(13)
		10·0			(14)
				2·0	(3)
Metho-trimeprazine	2·4	1·2	150	2·0	(4)
Chlorpromazine	1·0	1·0	1·0	1·0	
Trimeprazine	0·5	0·25	120	0·6	(5)
Promazine	0·47	0·5	0·5	—	(7)
Promethazine	0·34	0·1		0·25	(9)
			100		(10)
Perphenazine	0·25	0·25	1·0	0·05	(6)
Prochlorperazine	0·1	0·17	0·5	0·4	(12)

References continuous with Table 5. (13) Schmitt *et al.* (1957). (14) Brunaud *et al.* (1957a). The activity of all compounds is expressed in relation to chlorpromazine.

adrenaline and anti-tetanus activity. This is the only test in which acepromazine is 10 times as active as chlorpromazine. The anti-adrenaline activity of most other compounds is within a factor of two of their anti-tetanus activity. Some reduction in anti-adrenaline activity occurs in compounds which are powerful antihistamines, such as promethazine, trimeprazine and methotrimeprazine, all of which are over 100 times as active as chlorpromazine in this respect. A close correlation between anti-adrenaline and barbiturate potentiation has been reported by Brunaud, Schmitt, Aurousseau & Navarro (1957a & b) for a series of phenothiazine derivatives.

Some of the central effects of chlorpromazine can probably be explained by its adrenolytic activity. Adrenaline is known to produce electroencephalogram arousal (Bonvallet, Dell & Hiebel, 1954) and to facilitate spinal cord activity by an action on the brain stem (Dell, Bonvallet & Hugelin, 1954). Chlorpromazine has been shown to antagonize electroencephalogram arousal, whether induced by adrenaline (Hiebel, Bonvallet & Dell, 1954 ; Dell, Bonvallet & Hugelin, 1956 ; Bradley & Hance, 1957) or by nociceptive stimulation (Longo, Von Berger & Bovet, 1954 ; Rinaldi & Himwich, 1955 ; Bradley & Hance, 1957 ; and Unna & Martin, 1957) and to inhibit the facilitating effect of brain stem stimulation on spinal activity (Dasgupta & Werner, 1955 ; Henatsch & Ingvar, 1956).

Acepromazine is known to block the arousal response in a dose 1/6 to 1/10 of that of chlorpromazine (Schmitt, Mercier, Aurousseau, Hallot & Comoy, 1957). In addition, although chlorpromazine offers no protection against strychnine and leptazol it counteracts the convulsions produced by amphetamine and cocaine (Meidinger, 1956), both of which have central actions similar to adrenaline.

The ineffectiveness of meprobamate against experimental tetanus is rather surprising and not easily explained in view of its supposed muscle-relaxing activity and its apparent clinical value in tetanus (Perlstein, 1959). The fact that most of the information on this compound has come from one laboratory prompted Pfeiffer *et al.* (1957) to repeat some of the early pharmacological investigations. The

results of their experiments showed that meprobamate in fact possessed few of the properties of mephenesin. They preferred to classify it as a barbiturate with some of the properties of trimethadione, which is known to have no anti-tetanus activity (unpublished data).

A consideration of the difference in the effectiveness of mephenesin and meprobamate against strychnine and leptazol convulsions may help to explain the weak anti-tetanus activity of meprobamate. Compounds with relatively specific anti-strychnine activity are usually effective anti-tetanus drugs, probably because of the similar central actions of strychnine and tetanus toxin (a possible exception are the phenothiazine derivatives). Drugs which are especially effective against leptazol, however, are usually relatively weak anti-tetanus agents, unless they are capable of producing a widespread depression of the central nervous system.

Meprobamate is 5 times as active as mephenesin against leptazol and electroshock-induced convulsions, but only equally effective against strychnine convulsions (Berger, 1954 ; Pfeiffer *et al.*, 1957). Qualitative differences between the effects of these two compounds on strychnine and leptazol convulsions have also been observed. Pfeiffer *et al.* (1957) found that leptazol produced clonic convulsions when given after mephenesin but tonic extensor convulsions after meprobamate. In addition, meprobamate failed to raise the threshold to timed intravenous infusions of strychnine above 30%, whereas mephenesin, in smaller dose, raised it to above 100%. In this respect meprobamate resembled trimethadione. If anti-strychnine activity can be taken as a reliable guide of anti-tetanus potency these results suggest that mephenesin might possibly be more effective against tetanus than meprobamate.

Since both tetanus toxin and strychnine are thought to act primarily at the spinal level the ability of drugs to depress spinal reflexes should be a reliable guide to anti-tetanus activity. Mephenesin is twice as active as meprobamate in this respect (Pfeiffer *et al.*, 1957), but only half as active as 2-amino-6-methylbenzothiazole (Funderburk *et al.*, 1953). This order of activity is comparable to their anti-tetanus potency.

Another difference between the central action of mephenesin and meprobamate has been reported by Perlstein (1956), who found that, unlike mephenesin, which is of little value in the treatment of epilepsy, meprobamate is very effective against petit mal. This is a condition which is believed to originate from a focal disturbance of subcortical centres where electroencephalogram observation (Hendley *et al.*, 1955) suggests that meprobamate may act. Perlstein (1956) found that meprobamate produced no relief of spasticity in patients with pyramidal disorders.

These observations suggest that meprobamate may not be primarily a muscle relaxant of the mephenesin type. For, although it has been used with some apparent success in the relief of muscle tone in clinical tetanus (Perlstein, 1959), only those spasms induced by somatic stimuli such as noise, light or a pin-prick were controlled, whereas spasms triggered by visceral or propioceptive stimuli and which could be controlled with chlorpromazine were not suppressed (Perlstein, Stein & Elam, 1960).

Much of this work was performed whilst in receipt of a generous grant from May & Baker. I am much indebted to Dr D. R. Laurence for his helpful advice during discussion and also for the technical assistance of Miss S. Schadendorf.

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EFFECT OF L-TRYPTOPHAN ON DIURESIS AND 5-HYDROXYINDOLEACETIC ACID EXCRETION IN THE RAT

BY

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Oral administration of L-tryptophan to rats produced two main biochemical and pharmacological effects: a marked increase in urinary 5-hydroxyindoleacetic acid excretion, and a significant reduction in the urine flow after a water load. Urinary 5-hydroxyindoleacetic acid excretion reached its maximum 2 to 6 hr after the administration of tryptophan, and it increased with the dose of the amino acid. Antidiuresis was seen after the administration of L-tryptophan, 200 mg/kg, or more. The effect appeared promptly and it was roughly proportional to the dose of the amino acid administered. Both antidiuretic effect and increase in urinary 5-hydroxyindoleacetic acid excretion were more intense after oral than after parenteral administration of L-tryptophan. D-Tryptophan, in oral doses up to 1,000 mg/kg, produced neither an increase in urinary 5-hydroxyindoleacetic acid nor a reduction of diuresis. Available evidence suggests that reduction of urine flow is a consequence of biosynthesis and release of 5-hydroxytryptamine by the gastrointestinal mucosa. Tryptamine produced by direct decarboxylation of L-tryptophan does not seem to play any important role.

In the course of experiments on precursors of 5-hydroxytryptamine, it was observed that oral administration of L-tryptophan, the initial precursor of the amine, produced two main effects: a conspicuous increase in the urinary excretion of 5-hydroxyindoleacetic acid, and a significant reduction in the urine flow following a water load. The last effect seemed particularly worth studying, as it was caused in the normal animal by a common dietary amino acid.

The present experiments were undertaken to investigate which compound was responsible for the observed antidiuresis, in order to provide additional information on the physiological role of 5-hydroxytryptamine in the control of diuresis, as well as on the biosynthesis and turnover rate of the amine in the enterochromaffin cell system, which is generally considered the main site of production of 5-hydroxytryptamine.

METHODS

Experimental animals. Adult albino rats of both sexes weighing 170 to 230 g, all of the same breed, were used. They were kept on a standard laboratory diet. During the afternoon preceding each experiment they received a diet rich in water (soaked bread and vegetables); in the night the animals were deprived of food but allowed free access to water. On the next morning the animals were given tryptophan either orally (amino acid dissolved in 5 ml. tepid tap water/100 g of rat) or parenterally (amino acid dissolved in 3 ml. 0.9% sodium chloride solution/100 g of rat). Doses of the administered L-tryptophan varied from 50 to 1,000 mg/kg; those of D-tryptophan from 200 to 1,000 mg/kg. In some experiments the

parenteral administration of L-tryptophan was repeated 3 to 4 times in 24 hr, at an interval of 4 to 5 hr between two administrations. The same amount of tepid tap water or of 0.9% sodium chloride solution was given to the controls.

Urine collection and estimation of urinary 5-hydroxyindoleacetic acid. The rats were placed in diuresis cages in groups of 4 animals each. Urine was collected in graduated cylinders containing 0.3 ml. chloroform and 0.3 ml. acetic acid in order to preserve the excreted 5-hydroxyindoleacetic acid. The urine output was measured for 5 hr after giving water and tryptophan, at intervals of 30 to 60 min. The 5-hydroxyindoleacetic acid was estimated by the colorimetric method of Macfarlane, Dalgliesh, Dutton, Lennox, Nyhus & Smith (1956), on samples of urine taken 2, 6 and 24 hr after loads. Already from the first experiments it became clear that this method could not be applied to the urine of rats treated with either 1,000 mg/kg L-tryptophan or D-tryptophan, owing to the presence of large amounts of interfering substances. In this case 5-hydroxyindoleacetic acid was estimated semi-quantitatively by paper chromatography. Urine was extracted with peroxide-free ethyl ether at *p*H 3 to 4 and the concentrated ether phase was chromatographed using the ascending method on Whatman no. 1 paper. The solvent was the *n*-butanol-acetic acid-water mixture (4:1:5); the developing reagents were: (*a*) a 2% alcoholic solution of *p*-dimethylaminobenzaldehyde, followed by exposure in a large glass chamber to hydrogen chloride vapours; (*b*) Gibbs' reagent (a 0.05% alcoholic solution of 2-6-dichlorochinonchlorimide); and (*c*) Heinrich and Schuler's NNCD reagent (4-nitro-2-chloro-1-diazobenzene-α-naphthalene sulphuric acid).

Estimation of tryptamine and indole-3-acetic acid. Semi-quantitative estimation of tryptamine and indole-3-acetic acid was carried out by paper chromatography after purification of the urine on alumina column. A volume of 50 ml. of urine, both normal and from rats treated with 400 mg/kg L-tryptophan, was evaporated to dryness under reduced pressure at 40° C. The residues were dissolved in 100 ml. 96% ethanol and then passed through an alumina column 3.3 cm in diameter. The column of alumina (aluminium oxide Merck, according to Brockmann) weighed 100 g and reached a height of 15 cm. Elution was performed with progressively decreasing concentrations of ethanol. Each fraction of eluate (100 ml.) was concentrated and examined by paper chromatography with the technique previously described. Tryptamine was found to be present in the 80% alcoholic eluate, indole-3-acetic acid in the 70% and 60% alcoholic eluates. The semi-quantitative estimation was performed by visual comparison of the spots obtained with a series of spots containing known concentrations of the corresponding pure substances.

Indole compounds. The L- and D- forms of tryptophan were purchased from Hoffman-La Roche (Basle) and indole-3-acetic acid from Fisher Scientific Company (U.S.A.); 5-hydroxytryptamine and 5-hydroxyindoleacetic acid were kindly supplied by the Farmitalia Research Laboratories (Milan).

RESULTS

Effects of L-tryptophan and D-tryptophan on the diuresis of hydrated rats. The only pharmacological action produced by L-tryptophan was a reduction of the urine flow. This reduction was generally observed 1.5 hr after the water load and it was satisfactorily proportional to the dose of the amino acid administered. Results are shown in Table 1.

In rats treated with 50 and 100 mg/kg L-tryptophan, the reduction of diuresis was slight and not statistically significant, but in the groups of animals treated with 200 mg/kg L-tryptophan, the reduction was more conspicuous (14 to 30% less than the controls). It should be noted that, of the four groups of experiments carried out with a dose of 200 mg/kg, only two gave reductions that were highly statistically significant; in one the reduction was at the limits of significance; and in one it was

not significant. However, the average of all the values obtained from the groups treated with 200 mg/kg L-tryptophan was significantly lower ($P<0.05$) than that of the single control values.

As the dose of the amino acid was increased, the antidiuretic effect of L-tryptophan became more and more evident in both intensity and duration: a reduction of 38% was obtained with a dose of 400 mg/kg and one of 67% with 1,000 mg/kg of L-tryptophan. In this last instance, the reduction of the urine output was highly significant up to the 5th hr after the water load.

These results were all the more remarkable inasmuch as no action on diuresis was observed after oral administration of D-tryptophan, even at a dose of 1,000 mg/kg.

TABLE 1

ACTION OF L-TRYPTOPHAN AND OF D-TRYPTOPHAN ON RAT DIURESIS FOLLOWING WATER LOAD

The amounts of urine in ml./kg of body weight are shown, ±s.d. In parentheses the number of groups of rats is given; each group contained 4 animals. L-TP=L-tryptophan. D-TP=D-tryptophan

Tryptophan dose (L-TP or D-TP)	Urine (ml./kg) excreted after:		
	1½ hr	3 hr	5 hr
L-TP 50 mg/kg	35·2±3·2 (6)	46·8± 6·3 (6)	52·6±11·0 (6)
Controls	38·2±8·3 (6)	46·8±12·6 (6)	52·8±11·7 (6)
	$P>0.4$		
L-TP 100 mg/kg	33·8±4·2 (6)	47·3± 9·7 (6)	54·5± 3·4 (6)
Controls	38·2±8·3 (6)	46·8±12·6 (6)	52·8±11·7 (6)
	$P>0.2$		
L-TP 200 mg/kg	31·0±2·3 (5)	47·6±2·5 (5)	62·0± 4·2 (5)
Controls	44·0±6·3 (5)	56·0±3·4 (5)	62·6± 3·7 (5)
	$P<0.01$	$P<0.01$	
L-TP 200 mg/kg	27·5±4·1 (6)	38·5± 1·9 (6)	55·6± 3·2 (6)
Controls	35·5±2·3 (6)	41·2± 3·2 (6)	54·8± 4·1 (6)
	$P<0.01$	$0.2>P>0.1$	
L-TP 200 mg/kg	27·8±4·0 (6)		
Controls	32·5±3·1 (6)		
	$P>0.4$		
L-TP 200 mg/kg	25·7±4·3 (6)		
Controls	31·3±6·1 (6)		
	$0.1>P>0.05$		
D-TP 200 mg/kg	34·0±3·2 (7)	42·0±3·0 (6)	54·5±3·4 (6)
Controls	35·8±9·9 (14)	43·0±1·0 (6)	54·0±4·2 (6)
	$P>0.8$		
L-TP 400 mg/kg	18·3±4·1 (6)	34·7±4·3 (6)	46·1±2·5 (6)
D-TP 400 mg/kg	29·2±4·9 (6)	42·3±2·3 (6)	47·1±1·8 (6)
	$P<0.01$	$P<0.01$	
L-TP 1,000 mg/kg	10·0±3·2 (4)	20·5±5·4 (4)	40·7±8·0 (4)
Controls	30·5±5·5 (4)	46·5±4·2 (4)	55·0±7·0 (4)
	$P<0.01$	$P<0.01$	$P<0.01$
L-TP 1,000 mg/kg	8·4±2·2 (5)	23·2±4·9 (5)	36·0±2·9 (5)
D-TP 1,000 mg/kg	27·2±3·6 (5)	40·0±5·1 (5)	45·0±5·9 (5)
Controls	24·4±2·7 (5)	39·2±2·6 (5)	46·4±3·8 (5)

Action of L-*tryptophan on the urinary excretion of 5-hydroxyindoleacetic acid.* Observations on the urinary excretion of 5-hydroxyindoleacetic acid in the 24-hr period after an administration of L-tryptophan are shown in Table 2.

TABLE 2

URINARY EXCRETION OF 5-HYDROXYINDOLEACETIC ACID IN THE 24-HR PERIOD FOLLOWING ADMINISTRATION OF L-TRYPTOPHAN

The means ± s.d. of 5-hydroxyindoleacetic acid in μg/kg are shown. or. = orally; s.c. = subcutaneously; i.p. = intraperitoneally.

Dose of L-tryptophan	5-Hydroxyindole-acetic acid μg/kg	% increase	*P*
50 mg/kg, or.	138·2 ± 14·9 (5)	7	>0·2
Controls	129·0 ± 7·5 (5)		
100 mg/kg, or.	171·5 ± 17·2 (6)	35	<0·01
Controls	126·7 ± 11·7 (7)		
200 mg/kg, or.	221·2 ± 44·2 (19)	67	<0·01
Controls	133·5 ± 26·7 (22)		
400 mg/kg, or.	261·6 ± 64·0 (5)	106	<0·01
Controls	125·8 ± 7·3 (5)		
1,000 mg/kg, or.	400 to 500 (4)		
Controls	131·7 ± 12·0 (4)		
200 mg/kg, s.c.	163·6 ± 35·1 (10)	23	<0·05
Controls	132·8 ± 25·4 (10)		
200 mg/kg, i.p.	155·0 ± 11·6 (4)	37	<0·01
Controls	113·0 ± 5·8 (4)		

It clearly appears from Table 2 that all groups of rats treated with L-tryptophan showed an increase of urinary 5-hydroxyindoleacetic acid in comparison with the controls. When L-tryptophan was administered orally, this increase was barely evident and not significant with the dose of 50 mg/kg ; it became more conspicuous with higher doses ; with the dose of 400 mg/kg the amounts of 5-hydroxyindoleacetic acid excreted were twice those excreted by the control groups. Chromatographic estimation of 5-hydroxyindoleacetic acid after administration of 1,000 mg/kg L-tryptophan revealed values 3 to 4 times higher than the control values.

An increased excretion of 5-hydroxyindoleacetic acid, even if not so striking, was observed also in the groups of animals given L-tryptophan by the subcutaneous or intraperitoneal route.

In further experiments it was demonstrated that the effect on 5-hydroxyindoleacetic acid excretion of 3 to 4 successive doses of 100 mg/kg each, given subcutaneously at 4- to 6-hr intervals, did not significantly differ from that of a single dose of L-tryptophan 400 mg/kg.

In order better to establish the time of maximum output of urinary 5-hydroxyindoleacetic acid, this metabolite was estimated in some groups of rats also in the urine collected 2 and 6 hr after the tryptophan load. Results are shown in Table 3.

It can be seen that the time of highest excretion of 5-hydroxyindoleacetic acid after L-tryptophan load was that ranging between the 3rd and 6th hr. In this period 5-hydroxyindoleacetic acid excretion was 3 to 4 times higher than that of controls. A remarkable increase, though less conspicuous, was observed also in the following 18-hr period.

TABLE 3

URINARY EXCRETION OF 5-HYDROXYINDOLEACETIC ACID

The figures represent mean excretion of 5-hydroxyindoleacetic acid in μg/kg$\pm$s.d. In parentheses number of groups of rats; 4 animals in each group

Time in hr	Controls	Treated with L-tryptophan (200 mg/kg)
0–2	28·4$\pm$6·8 (5)	28·0$\pm$ 4·0 (5)
2–6	16·0$\pm$2·6 (5)	51·4$\pm$ 7·6 (5)
6–24	84·7$\pm$18·2 (8)	144·9$\pm$49·3 (8)

Chromatographic estimation of tryptamine and indole-3-acetic acid. Chromatographic estimation of tryptamine and indole-3-acetic acid gave the following values: (*a*) Normal urine: tryptamine undetectable, indole-3-acetic acid 300 μg/kg/24 hr. (*b*) Urine of rats given 400 mg/kg L-tryptophan by oral route: tryptamine 250 μg/kg/24 hr (recovery approximately 0.06%), indole-3-acetic acid approximately 4 mg/kg/24 hr (recovery roughly 1%).

DISCUSSION

Administration of L-tryptophan to rats produced two effects: a remarkable increase in the urinary excretion of 5-hydroxyindoleacetic acid, and a significant reduction of urine flow.

The observed increase in urinary 5-hydroxyindoleacetic acid is in accordance with the results obtained by other investigators in man (Lauer, Inskip, Bernsohn & Zeller, 1958; Kopin, 1959).

It is highly probable that the 5-hydroxyindoleacetic acid found in urine originates from L-tryptophan via 5-hydroxytryptophan and 5 -hydroxytryptamine. Alternative pathways, although possible from a theoretical point of view (L-tryptophan$\rightarrow$ tryptamine $\rightarrow$ 5-hydroxytryptamine $\rightarrow$ 5-hydroxyindoleacetic acid; L-tryptophan $\rightarrow$ indolepyruvic acid$\rightarrow$indole-3-acetic acid$\rightarrow$5-hydroxyindoleacetic acid; L-tryptophan $\rightarrow$5-hydroxytryptophan$\rightarrow$5-hydroxyindolepyruvic acid$\rightarrow$5-hydroxyindoleacetic acid), seem to be of no practical importance. The main site of 5-hydroxylation of L-tryptophan is in all probability the gastrointestinal mucosa. In fact, the increase in urinary 5-hydroxyindoleacetic acid obtained with oral L-tryptophan was greater than that obtained with similar subcutaneous or intraperitoneal doses of L-tryptophan. Moreover, it has been shown in previous experiments that in rats from which the whole gastrointestinal tract had been removed by operation, L-tryptophan given by subcutaneous route did not prevent the disappearance of urinary 5-hydroxyindoleacetic acid (Bertaccini, 1960).

Apparently the urinary output in 5-hydroxyindoleacetic acid increases with the dose of the administered L-tryptophan, and maximum excretion rate of 5-hydroxyindoleacetic acid occurs between the 2nd and the 12th hr following the administration of the amino acid.

A dose of 1,000 mg/kg L-tryptophan given by mouth produced a 300% increase in the 24-hr excretion of 5-hydroxyindoleacetic acid. It may therefore be inferred that 5-hydroxylation of L-tryptophan and biosynthesis of 5-hydroxytryptamine are

at least trebled following a tryptophan load. If under normal conditions the half-life of gastrointestinal 5-hydroxytryptamine in the rat is approximately 6 to 7 hr (Erspamer, 1955), after a tryptophan load it may be reduced to 2 hr. This means that under normal conditions the biosynthetic capacity of the enterochromaffin cells of the gastrointestinal mucosa are far from being saturated.

It has been found that the gastrointestinal tract of the rats commonly used in this laboratory weighs 40 to 50 g/kg body weight. The overall 5-hydroxytryptamine content of the gastrointestinal tract is approximately 2 to 4 μg/g and the daily urinary excretion of 5-hydroxyindoleacetic acid is 90 to 140 μg/kg. Assuming that the 5-hydroxytryptamine synthesized by the enterochromaffin cells is three times more than the 5-hydroxyindoleacetic acid excreted (Erspamer & Testini, 1959), it may be calculated from the data given above that under basal conditions the gastrointestinal tract synthesizes 5-hydroxytryptamine at a rate of 0.35 μg/g/hr, and that after a tryptophan load the rate of 5-hydroxytryptamine biosynthesis may be at least trebled for some hours, probably reaching or even surpassing values of 1 μg/g/hr.

Oral doses of 200 mg/kg L-tryptophan and, more evidently, larger doses produce a significant antidiuretic effect. D-Tryptophan is completely ineffective even with doses of 1,000 mg/kg. It is therefore certain that antidiuresis is produced by some metabolite which can originate solely from L-tryptophan. Practically only two metabolites may come into play: tryptamine and 5-hydroxytryptamine.

It is highly probable that 5-hydroxytryptamine is the main, if not the sole, agent responsible for antidiuresis. This claim is based on the following evidence. The amount of 5-hydroxytryptamine synthesized and released by the gastrointestinal mucosa following an oral tryptophan load is largely sufficient to cause antidiuresis. In fact, it has been repeatedly demonstrated that subcutaneous doses of as little as 4 to 10 μg/kg of 5-hydroxytryptamine produce a significant reduction of urine flow in hydrated rats, and that 5-hydroxytryptamine is 100 to 300 times more potent than tryptamine in reducing diuresis (Erspamer, 1954). Although the amount of tryptamine produced in the organism by decarboxylation of L-tryptophan cannot be calculated directly, strong indirect evidence supports the view that this amount is not sufficient to cause antidiuresis.

In this respect one has to consider that urine of rats given 400 mg/kg of L-tryptophan orally contains only 250 μg/kg tryptamine, whereas urine of rats given 400 mg/kg of DL-5-hydroxytryptophan (that is, 200 mg/kg L-5-hydroxytryptophan) contains as much as 33 mg 5-hydroxytryptamine (13 mg as free base and 20 mg as the glucuronide) (Erspamer & Bertaccini, 1962). This signifies that decarboxylation of L-tryptophan in the rat organism is at least 200 times less rapid than that of 5-hydroxytryptophan. Now, in spite of the easy attack by decarboxylase, the minimum oral dose of L-5-hydroxytryptophan causing a significant reduction of urine flow is as high as 100 mg/kg (Erspamer & Bertaccini, 1962), whereas the minimum oral dose of L-tryptophan active on diuresis is only twice as great. These results strongly suggest that tryptamine, which is produced from the precursor amino acid at a rate at least 200 times less than 5-hydroxytryptamine and which is 100 to 300 times less potent than 5-hydroxytryptamine, cannot be responsible for the antidiuresis provoked by L-tryptophan.

It may be observed that maximum antidiuretic effect appears during the first 1 or 2 hr following tryptophan load, whereas maximum excretion of 5-hydroxyindoleacetic acid is more delayed. This demonstrates that 5-hydroxytryptamine synthesis and liberation by the gastrointestinal tract follows very promptly the absorption of the precursor amino acid and provides additional proof that the amount of 5-hydroxytryptamine required to reduce urine flow is extremely small, as it is not detectable in urine in the form of excess 5-hydroxyindoleacetic acid. It may be that, as biosynthesis of 5-hydroxytryptamine continues, the released amine acts on the renal effectors less intensely, owing to the appearance of tachyphylaxis.

The production of an evident, easily repeatable pharmacological effect with a common dietary amino acid given by mouth is not an unusual phenomenon. It should be added that the antidiuretic effect of L-tryptophan was obtained in completely normal, non-pretreated animals.

It is difficult to decide whether this observation can be taken as evidence in favour of the hypothesis that 5-hydroxytryptamine may interfere in rats in the physiological control of diuresis. Certainly the circumstance that antidiuresis has been provoked by an excess of 5-hydroxytryptamine of endogenous origin (the remote precursor of endogenous 5-hydroxytryptamine is in fact L-tryptophan) is in agreement rather than in disagreement with such a hypothesis.

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TOXICOLOGICAL AND PHARMACOLOGICAL EFFECTS OF GADOLINIUM AND SAMARIUM CHLORIDES

BY

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A study has been made of the toxicology and pharmacology of gadolinium and samarium chlorides. The symptoms of acute toxicity following intraperitoneal injection are described. The chronic oral ingestion of both chemicals for 12 weeks produced no effects on growth or the blood picture, and only the male rats receiving gadolinium chloride showed liver damage. The pharmacological responses to both chemicals were mainly depressant on all systems studied, and death was associated with cardiovascular collapse coupled with respiratory paralysis. The greatest damage seen was on abraded skin, where non-healing ulcers were produced by both chemicals, whereas irritation of intact skin and ocular tissues was only transient in nature.

In the past many of the rare earth lanthanons were difficult to obtain, because methods were not available for their economical extraction from ores. Recent technological advances have remedied this situation, and many of the elements in this group are now produced in quantity (Spiller, 1960). However, little is known of their biological effects. Niccolini (1931) investigated the pharmacological effects of samarium on several systems. Durbin, Williams, Gee, Newman & Hamilton (1956) reported on the metabolism of the various lanthanons including Sm^{153} and Gd^{159}. They found that both of these elements concentrated in the liver and kidney and eventually were laid down in the skeleton although not to the same extent as the heavier members of the lanthanium group. The minimal lethal dose of $SmCl_3$ for rats was 2 g/kg by subcutaneous injection (Steidle & Durr, 1929; Niccolini, 1931) and for guinea-pigs 0.75 to 1.0 g/kg (Niccolini, 1931) or about 0.5 g/kg (Steidle & Durr, 1929). Kyker & Cress (1957) found gadolinium non-toxic in doses of 3.5 to 60 mg/kg of Gd by intravenous injection. Snyder, Cress & Kyker (1959) showed that samarium but not gadolinium produced fatty infiltration of the liver of rats receiving one intravenous injection of the chemical. In view of the fact that even the above information was not complete and because the purity of the salts used was not known, we have reinvestigated the toxicology and pharmacology of gadolinium and samarium with salts of known purity.

METHODS

The purity of the salts used was gadolinium chloride, 98%, and samarium chloride, 99%. The intraperitoneal LD50 figures were determined on 240 male CFI mice. The chronic toxicities of gadolinium and samarium chlorides were determined by including 0.01, 0.1 and 1% of either

compound in the diet and feeding it over a period of 12 weeks to 3 groups of rats for each compound. Each group contained 6 males and 6 females. Observations were made of the following: total erythrocytes, total leucocytes, differential cell count, haemoglobin, haematocrit and body weight. At the conclusion of the study the following tissues were examined histologically: heart, lung, liver, kidney, pancreas, spleen, adrenal and small intestine. The method of Draize, Woodward & Calvery (1944) was used to study ocular and skin irritation in rabbits and intradermal irritation in guinea-pigs. Three rabbits were used for each compound in the ocular studies; each rabbit had one eye exposed to 1 mg of crystals of gadolinium chloride or samarium chloride, while the other eye served as a control. In the rabbit skin irritation tests 6 animals were employed. Five guinea-pigs were used for each compound in the intradermal studies, and the concentrations of the chlorides were 1:10 to $1:10^6$.

Effects of gadolinium and samarium chlorides on isolated strips of guinea-pig ileum bathed in Locke solution were observed in a thermostatically regulated 25 ml. bath using the Trendelenburg method (1917). Experiments were also made on the isolated rabbit ileum in the presence of 0.5 μg of nicotine or 2.5 μg of acetylcholine.

Twenty cats of both sexes, weighing 1.79 to 4.08 kg, were anaesthetized with 0.5 ml./kg of "Dial"-urethane intraperitoneally. A six-channel Offner Dynagraph with Statham transducers was used to record carotid arterial pressure, respiration, nictitating membrane contraction, electrocardiogram lead II, femoral arterial pressure and femoral arterial flow. The arterial flow was measured with a 25 ml. Shipley–Wilson flowmeter (1951). Preganglionic stimulation of the cervical sympathetic fibres and the contralateral vagus fibres was carried out with a Grass model S-4 stimulator at 8 V/10 sec. Two hours were allowed to elapse before drug administration. Intravenous doses of the drugs used were: gadolinium chloride 0.5 to 50 mg/kg; samarium chloride 0.5 to 40 mg/kg; epinephrine 5 μg/kg; acetylcholine 5 μg/kg; histamine 0.5 μg/kg; and atropine 2 mg/kg. The gadolinium and samarium chlorides were injected at a constant volume of 1 ml./dose. Where appropriate, the results were analysed statistically by the Litchfield–Wilcoxon method (1949) or standard errors were determined.

RESULTS

Acute toxicity. The symptoms of acute toxicity for both gadolinium and samarium chloride were decreased respiration, lethargy, abdominal cramps and diarrhoea. The abdominal irritation forced the animals to crawl on their abdomens in order to move about the cage. Samarium also produced muscular spasms. The first deaths did not occur until 24 hr after injection. The peak mortality was reached between the fourth and fifth day. The intraperitoneal LD50/7 days was gadolinium chloride 550 (495.5 to 610.5) mg/kg and samarium chloride 585 (508.7 to 672.7) mg/kg and the respective slope values were 1.35 (1.08 to 1.69) and 1.36 (1.08 to 1.71). Oral administration of doses up to 2 g/kg produced no lethality, and as it was impossible to obtain more concentrated solutions further any evaluation of oral toxicity was not possible.

Chronic toxicity. Neither gadolinium nor samarium chloride had any apparent influence on the growth of rats of either sex throughout the feeding period of 12 weeks. Fig. 1 shows the growth curves for gadolinium chloride, and those for samarium are almost identical. Furthermore, both substances produced no significant effects on the haematology of the animals (see Table 1). The increases in the cells and haemoglobin between the beginning and the end of the experiment were probably related to normal growth pattern of the animals and not to any influence of the chemicals, because the control group did not differ significantly from the medicated groups. It should also be noted that all of the values in Table 1

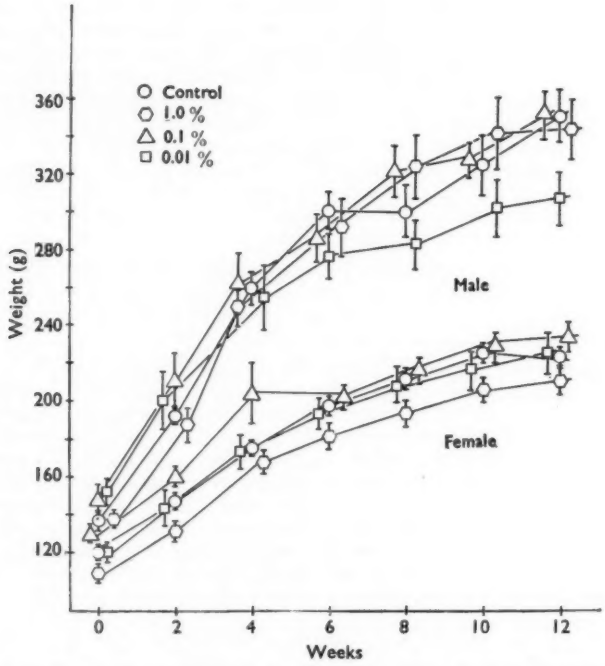

Fig. 1. Effects of various concentrations in the diet (0.01 to 1.0% w/w) of gadolinium chloride on growth in rats. The vertical lines represent the standard errors.

were within the ranges given for the rat by Gardner (1947). The data on the differential counts are not given in Table 1, because there was no significant difference between the medicated and control groups. At autopsy the internal organs of all groups of rats appeared normal and there were no outward signs of damage from ingestion of either gadolinium or samarium chlorides for 12 weeks. Gadolinium chloride produced perinuclear vacuolization of the parenchymal cells of the liver and a coarse granularity of their cytoplasm. Although these effects were not regularly observed at the 0.01 and 0.1% levels in the diet, all 6 male rats showed these changes at the 1% level. These histological effects were absent in the females at all levels. No changes were observed in the other tissues studied. Samarium chloride at all the concentrations used in the diet produced no histopathological changes in any of the organs studied.

Ocular irritation. The introduction of 1 mg of either gadolinium or samarium chlorides into the conjunctival sac of the eyes of rabbits resulted in an immediate increase in the rate of blinking and a redness of the palpebral conjunctiva within 1 hr. After 24 hr there was no evidence of corneal or iris damage, conjunctival irritation, chemosis or increased lacrimal discharge. Apparently the natural buffering capacity of the lacrimal fluid was sufficient to counteract the nascent hydrochloric acid released when the gadolinium or samarium chlorides dissolved in the tears.

TABLE 1

HAEMATOLOGICAL EFFECTS OF GADOLINIUM AND SAMARIUM CHLORIDES IN THE RAT

			Erythrocytes ($mm^3 \times 10^6$)		Leucocytes ($mm^3 \times 10^3$)		Haematocrit (vol. %)		Haemoglobin (g %)	
		Weeks:	0	12	0	12	0	12	0	12
Control	Male	Mean±s.e.	6·20±0·25	10·38±0·31	15·00±0·47	11·60±1·39	47±1·79	52±1·02	12·4±0·41	15·2±0·25
		Range	5·40–6·95	9·59–11·29	10·2–19·45	7·25–16·05	40–53	47–54	11·6–14·3	14·4–15·9
	Female	Mean±s.e.	6·51±0·32	8·99±0·38	13·20±0·58	18·20±2·02	47±1·07	50±0·80	12·6±0·25	13·9±0·24
		Range	5·08–8·35	7·67–11·00	7·00–18·60	10·6–30·80	43–54	47–54	11·3–13·6	12·5–15·2
Gadolinium 0·01%	Male	Mean±s.e.	6·12±0·18	9·89±0·52	13·70±2·01	13·70±2·56	43±1·03	51±1·65	12·5±0·18	15·0±0·53
		Range	5·60–6·70	7·87–11·70	8·25–14·85	12·60–24·00	39–46	45–57	11·8–13·0	13·5–16·6
	Female	Mean±s.e.	6·27±0·31	9·46±0·55	10·35±0·82	13·40±1·22	48±1·06	51±1·40	13·5±0·40	15·4±0·34
		Range	4·92–7·12	7·15–10·55	7·70–12·95	9·65–17·25	43–50	44–53	12·8–15·3	14·6–16·6
Gadolinium 0.1%	Male	Mean±s.e.	4·99±0·36	10·41±0·46	10·70±1·33	15·05±3·30	42±0·89	53±0·87	11·6±0·38	16·0±0·28
		Range	3·20–5·52	9·16–11·63	5·80–14·60	7·60–26·00	39–45	50–55	10·4–13·2	15·1–16·7
	Female	Mean±s.e.	5·79±0·19	8·67±0·34	8·90±0·74	13·30±1·28	46±1·28	49±0·88	13·0±0·16	15·1±0·35
		Range	5·00–6·40	7·59–10·05	7·05–11·20	9·45–18·80	42–49	46–50	12·6–13·5	13·4–15·7
Gadolinium 1.0%	Male	Mean±s.e.	6·45±0·51	10·61±0·48	10·10±0·75	13·20±1·19	46±1·66	54±0·71	12·4±0·40	15·8±0·25
		Range	5·08–7·95	9·22–11·95	8·40–12·60	9·80–16·80	40–50	51–55	10·8–13·7	14·6–16·2
	Female	Mean±s.e.	5·41±0·22	9·51±0·45	13·25±2·80	19·30±2·25	47±1·21	52±1·06	12·6±0·43	15·3±0·29
		Range	4·80–6·32	8·45–11·17	4·20–19·70	12·40–26·45	42–50	48–55	10·7–13·7	14·0–16·0
Samarium 0.01%	Male	Mean±s.e.	6·89±0·18	11·03±0·30	9·70±1·40	9·80±1·01	45±0·77	56±1·47	13·0±0·14	17·1±0·37
		Range	6·20–7·45	10·23–12·08	5·00–13·80	6·25–13·00	43–47	53–63	12·7–13·6	16·0–18·6
	Female	Mean±s.e.	6·80±0·23	9·55±0·28	11·05±0·57	11·30±0·92	49±1·90	50±0·80	13·2±0·31	15·0±0·33
		Range	6·25–7·80	8·39–10·19	8·65–12·40	9·00–14·15	45–58	47–53	12·5–14·2	13·9–16·2
Samarium 0.1%	Male	Mean±s.e.	6·53±0·29	9·89±0·17	13·20±1·18	10·60±1·25	47±1·00	51±0·48	12·8±0·25	14·7±0·20
		Range	5·90–7·75	9·35–10·48	10·10–16·75	7·40–15·0	43–49	49–52	12·2–13·9	14·3–15·6
	Female	Mean±s.e.	7·06±0·29	9·21±0·30	12·90±0·62	15·30±2·43	47±1·49	50±0·84	12·4±0·95	14·7±0·30
		Range	5·75–7·80	8·59–10·22	10·80–14·85	7·40–23·0	41–52	46–52	9·2–14·9	13·5–15·6
Samarium 1.0%	Male	Mean±s.e.	7·58±0·76	10·79±0·39	10·50±1·17	12·60±0·71	45±0·86	54±2·21	11·8±0·41	15·7±0·73
		Range	5·95–10·90	9·57–12·21	6·05–13·05	9·80–14·65	43–48	47–60	10·5–13·1	12·9–18·0
	Female	Mean±s.e.	6·47±0·45	10·13±1·23	9·90±1·47	12·50±1·46	47±1·03	50±1·54	12·4±0·31	14·5±0·41
		Range	5·45–8·05	9·08–16·59	7·45–16·10	7·65–17·80	44–51	47–57	11·0–13·0	13·5–16·4

Skin irritation. Direct application of gadolinium and samarium chloride crystals to intact rabbit skin produced no irritation within 24 hr and no delayed reaction after 72 hr. There was a very severe reaction to both chemicals by abraded skin resulting in the maximum irritation index of 8 within 24 hr. No change was observed within 72 hr, and within 7 days perforating ulcers 25 to 30 mm in diameter developed with penetration through the skin to the underlying muscle layers. Inasmuch as healing did not occur, the animals were killed at 14 days. The differences in response between intact and abraded skin may be related to the liberation of nascent hydrochloric acid by tissue fluids. Intradermal administration of gadolinium or samarium chlorides in guinea-pigs at concentrations of 1×10^{4} to 1×10^{6} gave 24 hr erythema indices of 1 (Draize *et al.*, 1944) and complete healing without scar formation within 7 days. Erythema, oedema and necrosis were observed 1 hr after injection of a 1 : 10 concentration of either chemical. The 24 hr irritation indices, erythema plus oedema formation, for concentrations of 1 : 10, 1 : 100 and 1 : 1,000 of both gadolinium and samarium chlorides were 8, 6 and 4. The three concentrations produced eschars whose diameters were 14, 10 and 6 mm for gadolinium and 12, 10 and 7 mm for samarium. Epilation of the area and scar formation occurred at 7 days with the 1 : 1,000 concentration of either chemical. At concentrations of 1 : 10 and 1 : 100, similar effects were observed after 14 days, but complete healing required 4 weeks. Such effects are probably related to the acidic nature of both compounds.

Effects on isolated intestine. An increasing depression of tonus and contractility of the rabbit ileum was produced by both gadolinium and samarium chlorides throughout the dosage range of 25 to 400 mg. When paralysis occurred at the highest dose, contractility of the intestine could not be restored by repeated washing. The spasmogenic effects of acetylcholine and nicotine were counteracted by the depressant action of both the rare earth elements. The antispasmodic ED50 figures for gadolinium and samarium chlorides against acetylcholine were 157 (74.8 to 329.7) mg and 187 (85 to 411.4) mg respectively and against nicotine were 360 (326.8 to 396.5) mg and 248 (138.6 to 443.9) mg. Similar depressant effects were observed with the Trendelenburg guinea-pig preparation where the ED50 figures for blocking both the circular and longitudinal muscular contractions respectively were: gadolinium 3.9 (2.17 to 7.02) mg and 5.8 (3.31 to 10.15) mg; samarium 2.4 (1.21 to 4.75) mg and 2.7 (1.56 to 4.51) mg. Because the muscular contractions of this preparation are induced by pressure stimulation of the enteric ganglia, it would appear that both gadolinium and samarium may produce intestinal depression by ganglionic blockade. However, experiments with the superior ganglion preparation of the cat indicated that this was unlikely.

Pharmacological effects. No observable pharmacological effects were produced in the anaesthetized cat by the injection of 5 to 10 mg/kg of gadolinium chloride or 5 to 15 mg/kg of samarium chloride. Administration of 20 to 25 mg/kg of either chemical produced transient hypotension of 15 to 60 mm Hg in both the carotid and femoral blood pressure, coupled with a decrease in femoral blood flow; the respiratory rate was unaffected. The dosage causing complete cardiovascular collapse was variable; gadolinium 30 to 50 mg/kg and samarium 35 to 40 mg/kg.

The terminal electrocardiographic changes included a transient increase in the height of the P-wave until it equalled the QRS complex, decreased height of the P-wave coupled with an increased T-wave, absence of the P-wave and QRS complex, inverted T-wave, notched T-wave, high take-off of the T-wave, 2 to 1 or 3 to 1 heart block and finally ventricular fibrillation. Within the dosage ranges studied, neither gadolinium nor samarium chlorides had an effect on the physiological responses to acetylcholine, epinephrine, histamine or vagal stimulation. Furthermore, neither chemical had any effect on transmission in the superior cervical ganglion or on contraction of the nictitating membrane. The cardiovascular effects of gadolinium and samarium chlorides could not be counteracted by epinephrine or atropine.

DISCUSSION

The present investigation of the toxicology and pharmacology of gadolinium and samarium confirms previous reports (Steidle & Durr, 1929 ; Niccolini, 1931 ; Kyker & Cress, 1957) and extends our knowledge of the biological effects of these chemicals. The failure to observe fatty infiltration of the liver following prolonged ingestion of samarium may have been related to rapid conversion of the chloride to the hydroxide and/or oxide and a failure of absorption of samarium in this form. Such an explanation appears likely because both Durbin *et al.* (1956) and Snyder *et al.* (1959) gave the chemicals by injection, thus by-passing the gastrointestinal tract. In both instances the substances were able to reach the liver and be excreted by the kidneys. The situation with gadolinium was different ; Snyder *et al.* (1959) reported no fatty infiltration of the liver, whereas we observed perinuclear vacuolization of the parenchymal cells and a coarse granularity of their cytoplasm. Although this histological change did not appear to affect the health of the animals since their growth and haematology were unimpaired, it is possible that more prolonged exposure to gadolinium chloride might cause detrimental changes. It is of interest that the CFN strain of rats used by Snyder *et al.* (1959) and also by us showed two different responses which appeared to be sex-linked, fatty livers being more consistent in females and parenchymal cell damage more consistent in males. The local effects on abraded skin present the greatest hazard in handling these chemicals, but such effects can be prevented by good industrial hygiene practice.

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EFFECTS OF INJECTION OF MEPESULPHATE INTO THE CEREBRAL VENTRICLES OF CATS

BY

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The injection of the anticoagulant mepesulphate (Treburon) into the cerebral ventricles of cats resulted in tremor, salivation, tachypnoea, loud calling, and brief periods of various forms of seizures followed by progressive reduction in spontaneous movements and somnolence. The possible sites of action for these effects are discussed.

Recently it was shown (Joseph, Jindal & Patel, 1959) that the anticoagulant mepesulphate (Treburon), when injected intravenously into dogs, rabbits and pigeons, greatly reduced the duration of anaesthesia induced by intravenous pentobarbitone. As mepesulphate had no analeptic action in unanaesthetized animals, the effect in the anaesthetized animals was suggestive of a specific antagonism to barbiturates.

The distribution of barbiturates in the brain following their administration has been the subject of many investigations. There is no selective localization in the mesencephalic and diencephalic areas, but a greater sensitivity of these structures to barbiturates is often alleged. As these areas are readily reached through the intraventricular route, the effects of mepesulphate injected into the cerebral ventricles of cats were examined with a view to obtaining information about the site and mode of its action in the central nervous system. The results were also expected to give an indication as to further lines of study of its possible barbiturate antagonism.

METHODS

Under pentobarbitone sodium anaesthesia, a Collison cannula was implanted aseptically into the left lateral ventricle of cats, as described by Feldberg & Sherwood (1953). After recovery from the operation, the cats were used for intraventricular injection once a week.

Mepesulphate, a heparin substitute, which is the sodium salt of sulphated polygalacturonic acid methyl ester methyl glucosamide, was kindly supplied by Hoffmann-la-Roche & Co., Switzerland. It is readily soluble, giving a clear and stable solution. For injection into the cerebral ventricles, it was dissolved in 0.9% NaCl solution and the volume injected was always 0.2 ml.

RESULTS

An intraventricular injection in cats of 5 to 10 mg of mepesulphate resulted in tremor, salivation, tachypnoea, loud calling, and various forms of seizure, often

* Deputed by the Government of Gujarat, India.

associated with mydriasis, salivation and pilo-erection. There was further a progressive reduction in spontaneous movements with varying depths of somnolence. This was a late effect and persisted for many hours after the seizures had ceased.

Tremor was an early effect which began 1 to 4 min after the injection. It was fine in character, was present in the ears, neck, trunk, fore- and hind-quarters, and occurred in bouts lasting usually for about half an hour. Twitching and flickering of the ears were also observed.

Salivation was not observed in all cats. When it occurred, it began 10 to 15 min after the injection and continued for 30 to 60 min. The saliva was usually thin and not copious; occasionally it became thick and mucoid. During the seizures salivation was often pronounced.

Tachypnoea was a regular effect which began 7 to 8 min after the injection, increased during the following 20 min and persisted up to 3 hr. Respiration became shallow and its rate increased to between 80 and 100/min. In the first hour or two of this tachypnoea, there were frequent periods of extremely quick and shallow respiration when the rate rose to 200/min; in one animal panting developed.

Loud calling was a regular feature. Three to five minutes after the injection the cat began miaowing, which within a short time developed into loud calling. The calling occurred in bouts which at first were infrequent and lasted for short periods only, but later became more frequent and prolonged. During seizures the calling ceased, but it was resumed immediately afterwards and was then particularly loud, each call being drawn out. At the late stage of reduced motor activity and somnolence, the periods of calling became less frequent and shorter; the calling itself became plaintive and feeble.

Seizures began 10 to 30 min after the injection. The cat became suddenly motionless, had a vacant stare, looked bewildered and did not react to auditory or visual stimuli. There was bending of the head sideways and downwards often associated with rocking movements, abrupt ear movements, and circling towards the side. During the circling the cat did not place its forepaws correctly, but placed the back of its paws on the floor. There was also twitching of the eyelids, and jactitation of the muscles of mastication resulting in snapping of the lower jaw. The twitching of the eyelids and of the facial muscles was usually more pronounced on the side towards which the head was bent. Bending and circling were unrelated to the side of cannulation of the lateral ventricle. After the head was bent, the cat remained sitting motionless for several seconds with a vacant stare. These events occurred in frequently recurring bouts, each bout lasting rarely more than half a minute. In several cats these bouts were the precursors of clonic contractions. Other forms of seizure activity were general convulsions and blind charging. Sniffing, hissing and snarling were seen occasionally.

The clonic contractions started in the hind leg of the side towards which the head was bent, lasting for 10 to 25 sec. They were first of small amplitude and executed from extreme flexion of the leg whilst the contralateral hind leg was extended. They spread to the foreleg of the same side, and, as the legs of the other side remained extended, the cat turned on its side and sometimes rolled over. As the clonic

contractions of the hind leg continued, they became more frequent, their amplitude became larger and the flexion decreased. Before the contractions ceased their frequency decreased and the last few contractions were executed from the fully extended leg. Such brief periods of clonic contractions occurred at intervals of a few minutes and continued for 1 to 3 hr. General convulsions lasting for a few seconds sometimes followed the clonic contractions ; at other times they occurred independently.

Blind charging was often preceded by a short period of extreme restlessness. Then the cat would suddenly charge ahead, running forward or springing upwards and sometimes clinging in an upright position to the side of the cage like a monkey. It would remain in this position for several seconds, staring vacantly. Such charging would be repeated 2 to 3 times in a single paroxysm.

Fig. 1. Cat placed in the palm of the hand 3 hr after an intraventricular injection of 5 mg mepesulphate.

During the various forms of seizure activity, the pupils were dilated, but as the seizures became less vigorous, pupillary dilatation became less conspicuous. Intense pilo-erection on the root of the tail and on the back and flanks occurred often during the general convulsions and the blind charging.

Reduction in spontaneous activity with somnolence was seen in all cats. It began half an hour after the injection, increased progressively for 1 to 2 hr and then persisted for several hours after the seizures had ceased. The cat lay in the cage in a drowsy or sleep-like condition, opening its eyes or holding up its head only on strong stimuli. Some cats curled up as in natural sleep. When the condition deepened, as it did in several cats, flexion of the limbs, particularly of the hind limbs,

became a characteristic feature. When induced to walk the cat did not unflex its legs but progressed or, sometimes, retrogressed, on its belly. When held by the scruff of the neck, the head drooped and the limbs hung without struggling. The righting and placing reflexes were impaired and the cat could not land properly when dropped. If one foreleg were gently placed backwards across its body, it would be maintained in this position for over a minute without being withdrawn. Muscle tone was greatly reduced, and when the cat was held in a hand it assumed the position shown in Fig. 1 without struggling or resisting. There was, however, some muscle tone and the cat was able to perform movements such as scratching the ears with the hind leg. Twenty-four hours after the injection, no residual effects were present and the cats appeared normal.

With injections of 2.5 mg of mepesulphate, all effects were attenuated or absent.

DISCUSSION

Drugs injected into the lateral ventricles through an indwelling cannula pass quickly into the third and fourth ventricles and then into the subarachnoid space. The effects of mepesulphate, therefore, could result from action on structures reached from the ventricular cavities or from the subarachnoid space. However, the pattern of reactions observed when analysed in conjunction with the effects obtained by previous workers on stimulation of areas of the diencephalon and of the amygdala and the hippocampus suggests that intraventricular mepesulphate acts on one or more of these sites.

Many of the effects produced by intraventricular mepesulphate resemble those produced by certain other drugs, when injected intraventricularly. Notable among these is tubocurarine, which also produces tremor, loud calling and seizures, with pupillary dilatation, salivation and pilo-erection. The striking resemblance is suggestive of common sites of action.

Tremor-like movements are produced in monkeys by electrical stimulation of the medial regions of the brain stem from the mesencephalon to the medulla (Jenkner & Ward, 1953), but in cats the tremor-evoking areas extend further rostrally to the hypothalamus (Birzis & Hemingway, 1957). It is likely that substances evoking tremor on intraventricular injection act as chemical excitants of these areas. Domer & Feldberg (1960), investigating tremor following administration of drugs into the cerebral ventricles of cats, excluded the areas in the medulla and caudal portion of the mesencephalon as the main sites of action since tremor was obtained when the drugs were perfused from the lateral ventricles to the middle of the aqueduct. It appears likely that mepesulphate also evokes tremor mainly by an action on the mid-line structures in the diencephalon and mesencephalon. Since these areas are the sites of highest 5-hydroxytryptamine concentration in the brain (Amin, Crawford & Gaddum, 1954), and since 5-hydroxytryptamine itself elicits tremor on intraventricular injection, Domer & Feldberg suggested that this amine may be involved in the regulation of tremor activity. The question thus arises whether drugs like mepesulphate which produce tremor on intraventricular injection act on these regions directly or through the release of 5-hydroxytryptamine.

The various forms of seizure following intraventricular mepesulphate may well result from an action on the hippocampus and/or amygdala. In support of this assumption are recent results obtained with tubocurarine. Feldberg & Fleischhauer (1961) observed that the seizure discharge, produced by its intraventricular injection and associated with the convulsive activity, results from an action on structures reached by penetration from the posterior half of the lateral ventricle. They concluded that the structures concerned are the hippocampus and/or amygdala. These structures are characterized by a low seizure threshold for electrical stimulation (Green, 1960). Moreover, chemical excitation by injection of acetylcholine with physostigmine or of carbachol into the hippocampus also produces a seizure discharge (MacLean, 1957). Electrical stimulation of the amygdala (Gloor, 1960) elicits not only the seizure discharge but also the autonomic effects—mydriasis, salivation, pilo-erection and hyperpnoea—which are the accompaniments of the seizure activity produced by mepesulphate and tubocurarine. These effects obtained in response to intraventricular mepesulphate may therefore result from an action on the amygdala as well. Loud calling is a feature of the action not only of mepesulphate but also of tubocurarine when given by the intraventricular route. Could it be that this " symptom " is also the result of an action on the amygdala ? The occasional vocalization (Nacquet, 1953) and frequent growling (De Molina & Hunsperger, 1959) observed on electrical stimulation of the amygdala would be in favour of this possibility. On the other hand, Hess (1954) found that loud calling was a frequent response to electrical stimulation of areas in the wall of the third ventricle from the level of the subthalamus above to the infundibulum below, and extending to the grey stratum of the aqueduct. Thus the loud calling observed with mepesulphate can result as well from its penetration into diencephalic and mesencephalic structures. An action on these structures would also account for the late stage of diminished spontaneous activity and somnolence since it is known that these structures are involved in the sleep-walking phenomena.

Drugs which elicit convulsions usually counteract the depressant effects of barbiturates without being specific competitive antagonists, but exerting a functional type of antagonism. These considerations apply also to the barbiturate antagonism of mepesulphate since the present experiments have shown it to be a convulsant on intraventricular injection. On systemic administration, a convulsive effect is observed only when a very large dose is given. In mice 5.5 g/kg was required on intravenous injection to induce convulsions (Mangieri, Engelberg & Randall, 1951). If the mechanism of action underlying these convulsions is the same as that responsible for the convulsions obtained on intraventricular injection with a dose as small as 5 mg, this would suggest that the blood-brain barrier has a low permeability to mepesulphate.

Mepesulphate is an anticoagulant of heparin-like structure. It would therefore be interesting to know whether heparin and its other substitutes when given by the intraventricular route would produce a pattern of reactions similar to that of mepesulphate.

I wish to thank Sir Charles Harington for hospitality, and Professor W. Feldberg and Dr K. Fleischhauer for their interest.

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EFFECT OF MORPHINE ON SOME SYMPATHETICALLY INNERVATED EFFECTORS

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Morphine, in doses of 0.5 to 55 mg/kg, inhibited contraction of the nictitating membrane of the cat following stimulation of the postganglionic sympathetic nerve fibres. Morphine was more effective at low than at high frequencies of stimulation, independently of the size of contraction of the membrane; the speed of contraction was reduced at all frequencies. Cocaine potentiated the contraction of the nictitating membrane following nerve stimulation more at low than at high frequencies, and antagonized the action of morphine. These findings, and the absence of an effect of morphine on the action of injected noradrenaline, make it likely that morphine interferes with the release of noradrenaline from the postganglionic nerve endings in the nictitating membrane. Morphine had no effect on the cardioaccelerator action of the cardiac nerves and inconsistent results were obtained on the emptying of the spleen after stimulation of the splenic nerves.

In recent years evidence has accumulated which shows that morphine inhibits the responses of certain autonomically innervated effectors. Thus in the isolated guinea-pig ileum it depresses the peristaltic reflex elicited by distending the lumen (Schaumann, 1955; Kosterlitz & Robinson, 1955, 1957), and in the rat and the rabbit it reduces the cardiac slowing produced by stimulating the right vagus nerve (Kosterlitz & Taylor, 1959a). Further, morphine inhibits the contraction of the nictitating membrane resulting from stimulation of the cervical sympathetic (Trendelenburg, 1957); it also diminishes the relaxing effect of sympathetic stimulation on the longitudinal muscle of the guinea-pig jejunum (Szerb, 1961).

Little is known about the mechanism of these actions of morphine, except that in guinea-pig ileum the release of acetylcholine is depressed after distension of the lumen (Schaumann, 1956) or coaxial electrical stimulation (Paton, 1956, 1957). In this paper an attempt has been made to analyse the action of morphine more thoroughly by studying its effect on the responses of the nictitating membrane, the sinoauricular node and the smooth muscle of the spleen to stimulation of their postganglionic sympathetic fibres. Some of the results have already been reported briefly elsewhere (Kosterlitz & Taylor, 1959b; Cairnie, Kosterlitz & Taylor, 1961).

METHODS

Adult cats of either sex were used. In most experiments the cats were anaesthetized by intraperitoneal injection (5 ml./kg) of a solution of 10% (w/v) urethane and 1% (w/v) chloralose, but when the cardiac nerves were stimulated, spinal preparations made by the

method of Kosterlitz, Krayer & Matallana (1955) were used. Since morphine depresses the respiratory centre, all experiments were carried out under artificial respiration.

In the experiments on the nictitating membrane, the left superior cervical ganglion and postganglionic trunk were exposed by the removal of the pharynx, larynx, upper part of the oesophagus and the long deep neck muscles. The postganglionic trunk was dissected out, secured by a fine ligature and severed from the ganglion, which was removed.

In the experiments on the heart rate, the approach to the cardiac nerves was through the first intercostal space. They were secured by a fine ligature just distal to the stellate ganglion which was then excised. Atropine (1 mg/kg) was injected intravenously to block the effect of stimulation of any aberrant vagal fibres.

In the experiments on the spleen, the abdomen was opened in the mid-line and the intestines were removed from the mid-duodenum to the terminal colon. The vascular connexions between the spleen and the stomach and omentum were tied and cut and the omentum removed. The splanchnic nerves were cut on both sides to prevent stimulation of the adrenal medullae. The splenic nerves were dissected away from the splenic artery, ligatured and cut.

For stimulation, the nerves were placed on perspex-embedded platinum electrodes and the area of dissection flooded with liquid paraffin. The nerves were stimulated with biphasic rectangular pulses from a Grass stimulator, using an RF-coupled output stage to isolate the stimuli from earth. Unless otherwise stated, the stimuli were supramaximal, and were applied for 45 sec every 3 to 4 min to the cervical postganglionic trunk, for 1 min every 4 to 5 min to the cardiac nerves and for 90 or 105 sec every 5 to 10 min to the splenic nerves.

The contractions of the nictitating membrane were recorded isotonically with a frontal writing lever and the change in the volume of the spleen by means of a plethysomograph chamber and piston recorder. The resting heart rate was determined from the electrocardiogram for a period of 10 sec at the beginning of each successive minute; when the cardiac nerves were stimulated, the heart beats were counted from the 15th to the 25th and the 45th to the 55th sec of the period of stimulation.

When stimulus frequency/response curves were determined, the contraction of the nictitating membrane or the decrease in volume of the spleen was plotted as a percentage of the maximal response against the logarithm of the stimulus frequency. Before the administration of morphine two control curves were obtained. Then morphine was injected and the series of observations was repeated. In the experiments on the heart rate, stimulus frequency/response curves were not constructed. In any one such experiment, we used the same frequency throughout, so chosen as to give an increase in heart rate of about 50% of the possible maximum.

The blood pressure in the left femoral artery was measured by means of a condenser manometer. All injections of drugs were made into the right femoral vein.

The doses of morphine hydrochloride, nalorphine hydrochloride, bretylium tosylate and dibenyline (phenoxybenzamine) hydrochloride refer to the salts, those of noradrenaline to the base.

RESULTS

Contraction of the nictitating membrane

Stimulus frequency/response curves. These curves, which were obtained by applying supramaximal stimuli at different frequencies to the postganglionic nerve fibres, were sigmoid in shape. In most preparations the response was just noticeable at a frequency of 0.125 stimuli/sec, and the maximum response was obtained at a frequency of 16 or 24 stimuli/sec (Fig. 1). Any one curve was reproducible within narrow limits for periods of 1 to 2 hr.

At the lowest frequencies (0.125 to 0.25 stimuli/sec) the fusion of the contractions was incomplete, and in particularly sensitive membranes, stimulated at 0.0625 stimuli/sec, a discrete contraction was observed in response to each stimulus.

Effect of morphine on the stimulus frequency/response curve. In all the 15 experiments in which morphine was given after control stimulus frequency/response curves had been established, small doses reduced the contractions of the nictitating membrane. This effect was much greater at low than at high frequencies (Fig. 1). The onset of inhibition was very rapid, occurring within 1 min after injection.

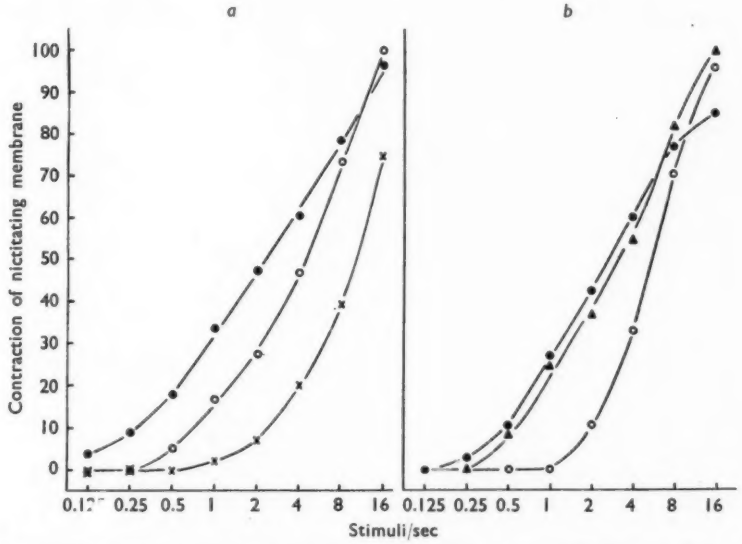

Fig. 1. Stimulus frequency/response curves showing the effects of morphine alone and of nalorphine given subsequently on the contractions of the nictitating membrane of the cat when the post-ganglionic nerve fibres were stimulated supramaximally. Abscissa: frequency of stimulation. Ordinate: contraction of the nictitating membrane as a percentage of the maximal contraction. a: ●——●, control; ○——○, after 1 mg/kg morphine; X——X, after additional 10 mg/kg morphine. b: ●——●, control; ○——○, after 15 mg/kg morphine; ▲——▲, after subsequent injection of 7.5 mg/kg nalorphine.

There was a considerable variation in the degree of inhibition found in different cats given the same dose of morphine, and, in any one cat, large doses were relatively less effective than small doses. For example, after doses of morphine of 0.5 to 2 mg/kg body weight, the response to 2 stimuli/sec was reduced by an average of 55 to 60%, the variation being from 33 to 76% in all experiments. However, there was a decrease of only 70% (range 68 to 73% in 3 experiments) after doses as large as 40 to 55 mg/kg body weight.

The morphine antagonist, nalorphine, reversed the effect of morphine either partly or, in some experiments, completely (Fig. 1b).

Correlation of the magnitude of the morphine effect with either the size of the contraction or the frequency of stimulation. The greater effectiveness of morphine in reducing the size of contractions produced by stimulation at low frequencies could be associated with either the smaller size of the response under these conditions (Trendelenburg, 1957) or with the frequency of stimulation. These alternative possibilities were tested by constructing stimulus frequency/response curves for supramaximal and submaximal stimuli and examining the effect of morphine on them.

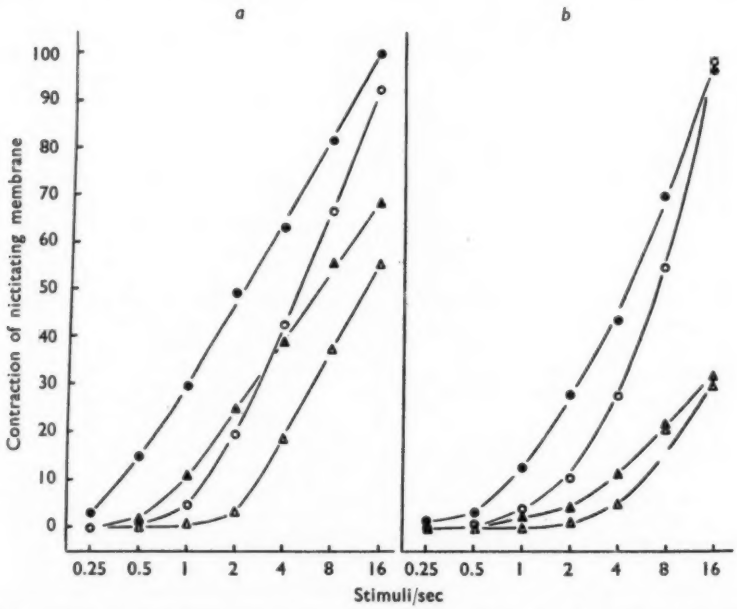

Fig. 2. Stimulus frequency/response curves showing the effects of morphine on the contractions of the nictitating membrane of the cat when the postganglionic nerve fibres were stimulated supramaximally and submaximally. The strength of the submaximal stimulus was chosen to give at 16 stimuli/sec a contraction of about 70% (a) and 30% (b) of the supramaximal stimulus. Abscissa: frequency of stimulation. Ordinate: contraction of the nictitating membrane as a percentage of the maximal contraction. ●——●, control, supramaximal stimulation; ▲——▲, control, submaximal stimulation; ○——○, after 2 mg/kg morphine, supramaximal stimulation; △——△, after 2 mg/kg morphine, submaximal stimulation.

The submaximal stimuli used were such as to give, at a frequency of 16 stimuli/sec, responses of either 70 or 30% of those obtained at this same frequency with the supramaximal stimuli. The effect of reducing the strength of the stimulus was to diminish the responses at high as well as at low frequencies (Fig. 2).

The effect of morphine on the responses obtained with submaximal, as with supramaximal, stimulation was greater at low than at high frequencies (Fig. 2). Or, to put it differently, when the size of the control response at 16 stimuli/sec was,

as a result of submaximal stimulation, only 30% of that obtained with supramaximal stimulation at this frequency, morphine had no inhibitory effect (Fig. 2b). On the other hand, a contraction of this same size produced by supramaximal stimulation at 2 stimuli/sec was reduced to one-third by the same dose of morphine. Therefore the effect of morphine was not correlated with the size of the contraction but with the frequency of stimulation.

Effect of morphine on the contractions of the nictitating membrane due to injected noradrenaline. Trendelenburg (1957) showed that morphine did not reduce the small contractions caused by injection of small doses of noradrenaline. It was, however, of importance to make quite sure that morphine had no inhibitory effect

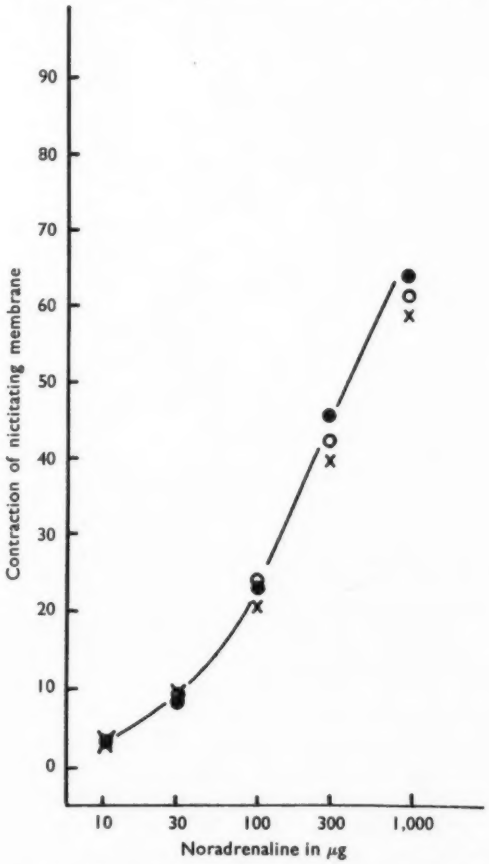

Fig. 3. Dose/response curves showing the effect of morphine on the contraction of the nictitating membrane of the cat resulting from the intravenous injection of noradrenaline. Abscissa: dose of noradrenaline. Ordinate: contraction of the nictitating membrane as a percentage of the maximal contraction obtained by electrical stimulation of the postganglionic fibres. ●—●, control; ○—○, after 2 mg/kg morphine; X—X, after additional 3 mg/kg morphine.

on contractions caused by larger doses of noradrenaline. By injecting into a cat of 2.2 kg doses of noradrenaline varying between 0.01 and 1 mg, a dose/response curve was constructed which included sizes of contractions of between 5 and 60% of the size of the maximal contraction obtainable by stimulation of the nerve (Fig. 3). Morphine in doses up to 5 mg/kg had no effect on the responses of the membrane to doses of between 0.01 and 0.1 mg noradrenaline, whereas those to 0.3 and 1 mg were reduced by only 13 and 18% respectively.

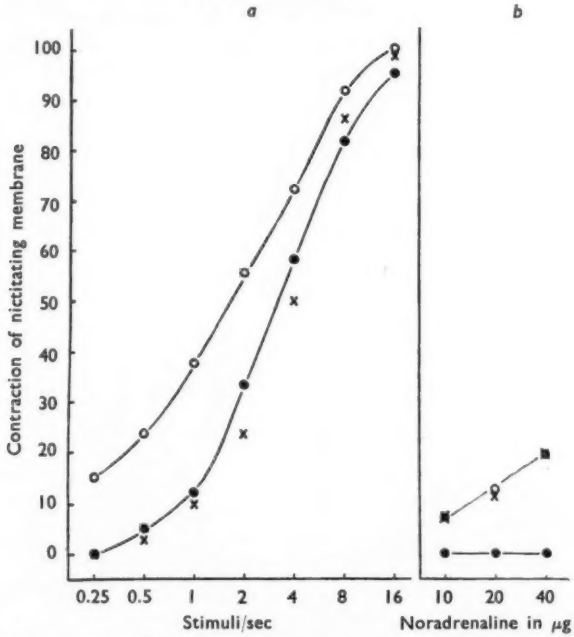

Fig. 4. The effect of cocaine followed by morphine on the contractions of the nictitating membrane of the cat produced by (a) supramaximal stimulation of the postganglionic nerve fibres and (b) intravenous injection of noradrenaline. Abscissa: (a) frequency of stimulation; (b) dose of noradrenaline. Ordinate: contraction of the nictitating membrane as a percentage of the maximal contraction. ●——●, control; ○——○, after 2 mg/kg cocaine; X——X, after subsequent injection of 1 mg/kg morphine.

Relationship between the effects of morphine and cocaine on the stimulus frequency/response curve. Cocaine, which is known to increase the sensitivity of the nictitating membrane to injected noradrenaline, was more effective in potentiating the responses to stimulation at low than at high frequencies (Fig. 4a). When morphine was given after sensitization of the membrane by cocaine, it caused the stimulus frequency/response curve to return to the level of the control curve, but it did not affect the responses to injected noradrenaline (Fig. 4a and b). When, on the other hand, morphine was given first, cocaine in suitable doses almost restored the stimulus frequency/response curve to normal (Fig. 5). The potentiating effect of cocaine, greater at low frequencies of stimulation, was not correlated with the

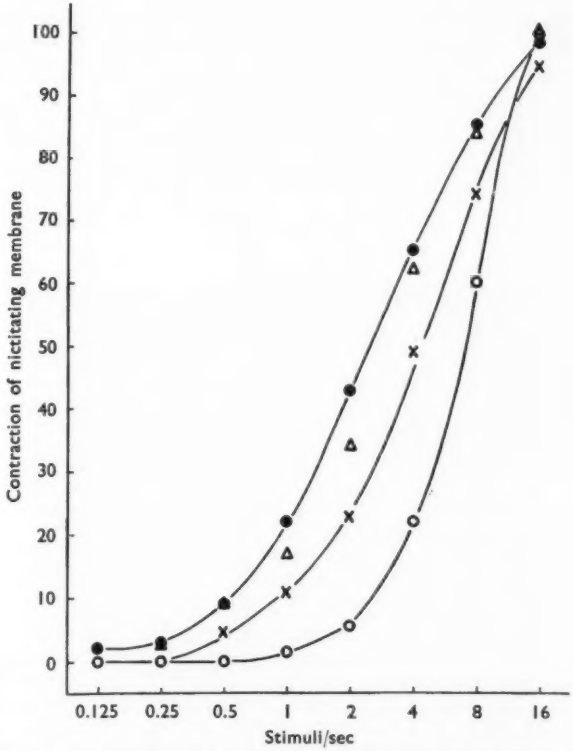

Fig. 5. The effect of morphine followed by cocaine on the contractions of the nictitating membrane of the cat produced by supramaximal stimulation of the postganglionic nerve fibres. Abscissa: frequency of stimulation. Ordinate: contraction of the nictitating membrane as a percentage of the maximal contraction. ●——●, control; ○——○, after 2 mg/kg morphine; X——X, after subsequent injection of 0.4 mg/kg cocaine; △——△, after additional 0.4 mg/kg cocaine.

size of the contraction. This was shown by the finding that sensitization of the membrane by increasingly large doses of cocaine produced proportionate and parallel shifts to the left of the noradrenaline dose/response curve, at least until the contractions were from 40 to 50% of the size of those caused by maximal electrical stimulation (Fig. 6).

When the postganglionic nerve fibres to a nictitating membrane, sensitized by administration of cocaine, were stimulated at a very low frequency, about 4 stimuli/min, a discrete contraction was observed for each stimulus. Morphine abolished this response completely (Fig. 7).

The effect of morphine on the rate of contraction of the nictitating membrane. Morphine increased the latent period and reduced the speed of contraction of the nictitating membrane due to postganglionic stimulation at low frequencies (Fig. 8a and b). This was not solely due to the decrease in the size of the contraction,

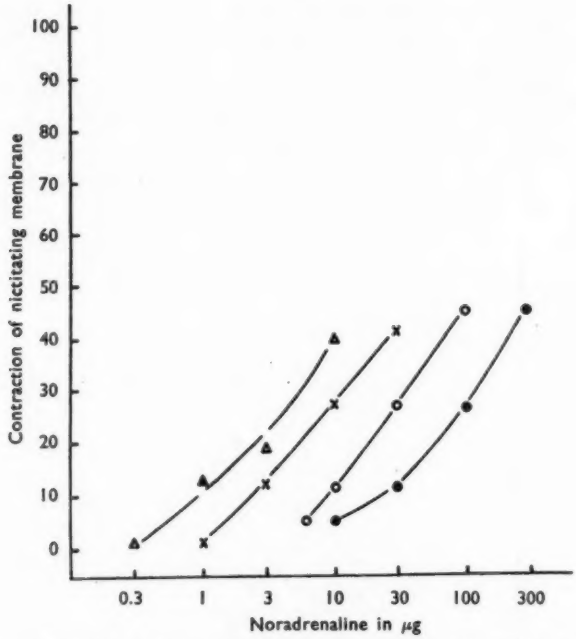

Fig. 6. The effect of cocaine on the responses of the nictitating membrane of the cat to intravenous injection of noradrenaline. Abscissa: dose of noradrenaline. Ordinate: contraction of the nictitating membrane as a percentage of the maximal contraction obtained by electrical stimulation of the postganglionic fibres. ●——●, control; ○——○, after 0.3 mg/kg cocaine; X——X, after additional 0.6 mg/kg cocaine; Δ——Δ, after additional 1.8 mg/kg cocaine.

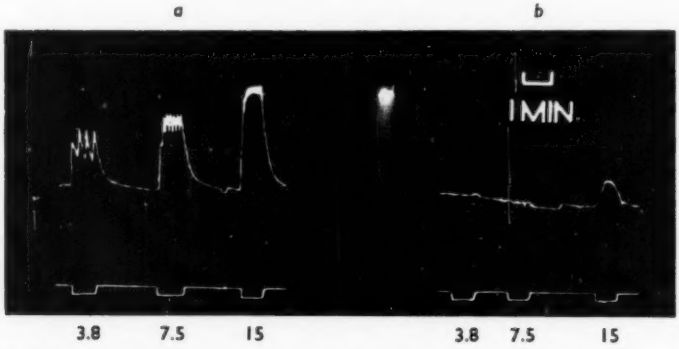

Fig. 7. The effect of morphine on the responses of the nictitating membrane of the cat to supramaximal stimulation at very low frequencies of the postganglionic nerve fibres. The membrane was sensitized by 2 mg/kg cocaine. (a) control; (b) after 2 mg/kg morphine. Numbers indicate stimuli/min.

since a similar reduction in size brought about by submaximal stimulation had much less effect on the speed of contraction (Fig. 8c). Although at high frequencies morphine reduced the height of contraction only very little, it was rather more effective in diminishing the speed of contraction (Fig. 8d and e).

Effects of bretylium and dibenyline on the frequency of stimulus/response curve. In view of the striking effect of morphine on the stimulus frequency/response curve, it was of interest to compare the effect of morphine with that of other drugs which reduce the responses of the nictitating membrane to nerve stimulation.

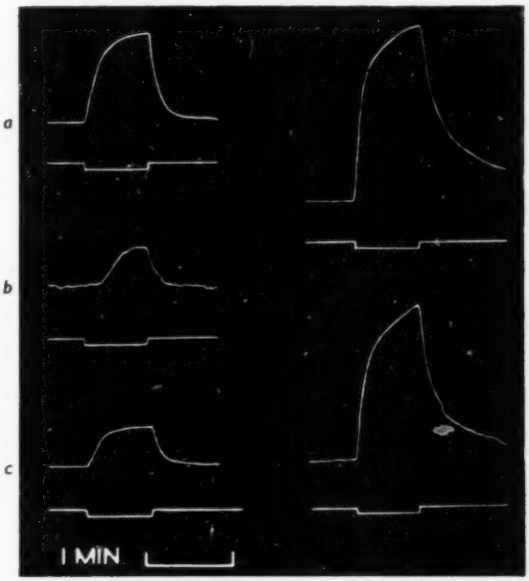

Fig. 8. The effect of morphine on the rate of contraction of the nictitating membrane of the cat following stimulation of the postganglionic nerve fibres. (a) control, 1 stimulus/sec, supramaximal; (b) after 2 mg/kg morphine, 1 stimulus/sec, supramaximal; (c) control, 4 stimuli/sec, submaximal; (d) control, 10 stimuli/sec, supramaximal; (e) after 2 mg/kg morphine, 10 stimuli/sec, supramaximal.

Bretylium, which, like morphine, does not depress the action of injected noradrenaline on the nictitating membrane (Boura & Green, 1959), depressed the contractions more at high than at low frequencies; indeed, in doses up to 1.4 mg/kg, it had no effect on the contractions produced by stimulation at frequencies between 0.125 and 0.5 stimuli/sec (Fig. 9a).

Dibenyline, which antagonizes the action of noradrenaline on the nictitating membrane, reduced the responses at high as well as at low frequencies of stimulation (Fig. 9b).

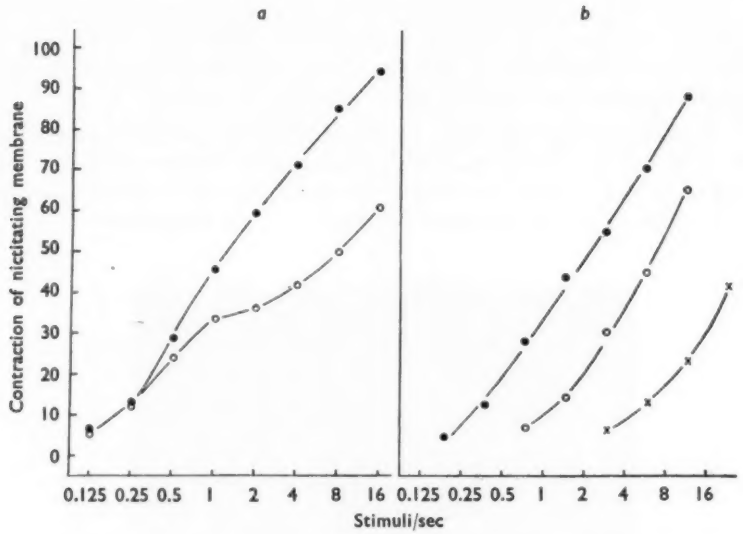

Fig. 9. Stimulus frequency/response curves showing the effects of (a) bretylium and (b) dibenyline on the contractions of the nictitating membrane of the cat produced by supramaximal stimulation of the postganglionic nerve fibres. Abscissa: frequency of stimulation. Ordinate: contraction of the nictitating membrane as a percentage of the maximal contraction. (a) ●——● control; ○——○, after 1.4 mg/kg bretylium. (b) ●——●, control; ○——○, 43–58 min after 0.3 mg/kg dibenyline; X——X, 30 min later.

Contraction of the spleen

It was impossible, in the course of 7 experiments, to demonstrate a constant effect of morphine on the contraction of the capsule of the spleen caused by stimulation of the splenic nerves. For instance, in one experiment, morphine inhibited the responses to electrical stimulation but the contractions caused by injection of noradrenaline were also reduced. In another experiment, after injection of morphine, there was a slight increase in the size of the responses to both stimulation of the splenic nerves and injection of noradrenaline. The morphine antagonist, nalorphine, depressed both types of response in one experiment, but in the other the results were equivocal.

Thus, although in the experiments on the nictitating membrane morphine clearly depressed the response to nerve stimulation but had no effect on the responses to injected noradrenaline, no such distinction could be shown to hold for the spleen.

Acceleration of the heart beat

In 8 experiments on the increase in heart rate brought about by the stimulation of the right cardiac nerves, no inhibitory effect of morphine was found (Fig. 10).

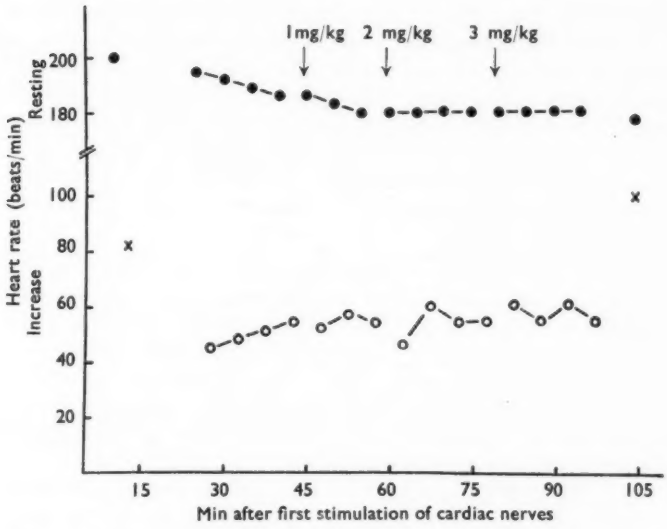

Fig. 10. The effects of morphine on the resting heart rate and on the increase in heart rate of the cat resulting from supramaximal stimulation of the cardiac nerves. Abscissa: time after first stimulation of the cardiac nerves. Ordinate: top, resting heart rate; bottom, increase in heart rate on stimulation. ●—— ●, resting heart rate; ○—— ○, increase in heart rate after stimulation at 1.4 stimuli/sec; X, increase in heart rate after stimulation at 15 stimuli/sec (maximal effect). At the arrows, the indicated amounts of morphine were injected intravenously.

DISCUSSION

We have examined the effects of morphine on the responses of three different sympathetic effectors to postganglionic stimulation. There was inhibition of the contraction of the nictitating membrane, but there was no effect on the cardio-accelerator action of the cardiac nerves. It was doubtful if there was any action on the emptying of the spleen after stimulation of the splenic nerves. This inconsistency in the effect of morphine is in keeping with what has already been pointed out for certain parasympathetic effectors: morphine has an inhibitory effect on the vagal slowing of the heart in the rabbit but not in the guinea-pig, while the peristaltic reflex is suppressed in the guinea-pig but not in the rabbit (Kosterlitz & Taylor, 1959a).

There are a number of possible ways in which morphine might exert its inhibitory action. We have not yet investigated the possibility of an effect of morphine on impulse propagation in the postganglionic fibres innervating the nictitating membrane. Morphine, however, causes no changes in the conduction velocity in the A-B and C groups of fibres of the rabbit vagus nerve and the α, δ and C fibres of the cat saphenous nerve. Also, there is no delay in the recovery of excitability after a single conditioning stimulus in α and δ fibres of the cat saphenous nerve (Cairnie & Kosterlitz, 1961). Since the effect of morphine on the responses of the

nictitating membrane is greatest at low frequencies of stimulation, there would have to be a profound change in impulse propagation to account for our observations on the effect of morphine on the shape of the stimulus frequency/response curve. Indeed, only a more or less complete block of conduction could explain the suppression by morphine of the discrete responses to single stimuli seen in the membranes sensitized by cocaine. Further, it has been shown that when, by applying submaximal stimuli, a smaller number of nerve fibres is activated, the effect on the stimulus frequency/response curve is very different from the change observed after administration of morphine.

These findings, and the absence of an effect of morphine on the action of injected noradrenaline, seem to make it likely that morphine interferes with the release of noradrenaline from the postganglionic nerve endings in the nictitating membrane, a suggestion already made by Trendelenburg (1957). This is in agreement with the results obtained by Thompson (1960) on the isolated nerve-muscle preparation of the nictitating membrane ; he found that morphine depressed the responses to supramaximal nerve stimulation but did not affect the contractions produced by adding adrenaline or noradrenaline to the bath fluid. So far, however, there is no direct evidence for such a diminution of noradrenaline release. The only site where morphine has been shown to reduce the amount of transmitter released is the cholinergically innervated smooth muscle of the guinea-pig ileum (Paton, 1956, 1957 ; Schaumann, 1956).

An important feature of the action of morphine is the fact that the reduction in the size of the contractions is greatest at low frequencies. A similar observation has already been made about the effect of morphine on the response of the sinoauricular node to stimulation of the right vagus nerve in the rabbit (Kosterlitz & Taylor, 1959a). Morphine differs in this respect from drugs which antagonize the action of the transmitter on the autonomic effector organ. The effect of such antagonists is always at least as great at high frequencies as at low frequencies. This is illustrated by the action of dibenyline on adrenergic neurones in the present series of experiments, and by that of atropine on the cholinergic neurones of the vagus (Kosterlitz & Taylor, unpublished observations).

Another observation which may be characteristic of the action of morphine on autonomic effectors is the decrease in the speed of contraction of the nictitating membrane, which is found not only at low but also, less obviously, at high frequencies of stimulation. It may be then that morphine exerts an inhibitory effect at these high frequencies although it does not reduce the size of contraction finally reached after 45 sec of stimulation. This may be in line with the findings of Kosterlitz & Taylor (1959a) that, although at high frequencies of stimulation morphine did not affect the degree of cardiac slowing resulting from stimulation of the vagus, there was a marked delay in the onset of the slowing.

There is a discrepancy between the conclusions drawn by Trendelenburg (1957) and ourselves about whether the effect of morphine is correlated with size of contraction, as he thought, or with frequency of stimulation. This probably arises from differences in experimental procedure. Trendelenburg stimulated for only 5 sec while we stimulated for 45 sec in order to be sure that at any given strength

and frequency of stimulation the maximum contraction was in fact obtained. A period of 5 sec is insufficient for this purpose, especially after morphine has been given, in view of its depressing effect on the speed of contraction.

Until the release of transmitter has been measured directly, we cannot know whether morphine reduces the amount liberated per volley to the same extent at low as at high frequencies of stimulation. Any attempt to explain more completely the effect of morphine on the stimulus frequency/response curve must remain speculative.

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